

THE URINARY EXCRETION OF CONJUGATED  
GLUCURONIC ACID IN HEALTHY MALE VOLUNTEERS

by

Peter Stephen Murano

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

APPROVED:

R.E. Webb, Chairman

F.W. Thye

B.M. Anderson

August, 1986

Blacksburg, Virginia

THE URINARY EXCRETION OF CONJUGATED  
GLUCURONIC ACID IN HEALTHY MALE VOLUNTEERS

by

Peter Stephen Murano

Christiansburg, VA 24073

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

APPROVED:

R.E. Webb, Chairman

F.W. Thye

B.M. Anderson

August, 1986

Blacksburg, Virginia

THE URINARY EXCRETION OF CONJUGATED  
GLUCURONIC ACID IN HEALTHY MALE VOLUNTEERS

by

Peter S. Murano

Committee Chairman: Ryland E. Webb  
Human Nutrition and Foods

(ABSTRACT)

The amount of urinary conjugated glucuronic acid excreted by a free-living male population and the effect of certain factors, i.e. vegetable, fruit, meat, and charbroiled food intake, tobacco, alcohol, caffeine, and marijuana use, exposure to chemicals and familial cancer incidence, were investigated. Urine was collected for 24 hours from 117 subjects who complied with the collection protocol and analyzed for each subject. Three days of urine were analyzed for a randomly selected subgroup of forty subjects.

For the one-day sample, the mean conjugated glucuronic acid excreted was 0.725 mmole/24 hr or 0.0492 mole/mole creatinine. The values for the three-day sample were 0.848 mmole/24 hr or 0.0562 mole/mole creatinine.

An analysis of the one-day data revealed a large degree of between-subject (inter-) variability: 47.1%. The corresponding coefficient of variation for the three-day

data was 48.2% when three day averages were compared. The within-subject (intra-) variability for the three-day data corresponded to a coefficient of variation of 29.2%.

The large intervariability probably masked any effects of diet, environment, or genetics upon the observed urinary conjugated glucuronic acid excretion. Caffeine use, vegetable, and fruit intake did show differences between low, moderate, and high consumers, although the biological importance of these associations for the small sample sizes is questionable. Further research regarding conjugated glucuronic acid excretion and dietary, environmental, and genetic influences is therefore warranted.

## ACKNOWLEDGEMENTS

I sincerely express my gratitude to Doctors Ryland Webb, Forrest Thye, and Bruce Anderson for serving on my graduate committee and for being true sources of inspiration to me in this undertaking.

I gratefully acknowledge the technical advice and assistance provided by Carolyn Harris and Leslie Reynolds, and to my colleagues Jane Santi and Vernice Robichaud.

Special thanks are due Ann Giovannitti-Jensen for the statistical analysis and her tireless patience.

Most of all I praise the support and assistance given me by my wife Elsie; to her, to my parents, and to my Lord this thesis is dedicated.

## TABLE OF CONTENTS

Chapter	Page
I. INTRODUCTION AND OBJECTIVES.....	1
II. REVIEW OF LITERATURE.....	5
Conjugation of Xenobiotics .....	5
Background Information.....	5
The Eight Classical Conjugation Reactions.....	7
Competitive Conjugation .....	12
The Metabolism of Glucuronic Acid.....	14
Glucuronic Acid Biosynthesis.....	14
Glucuronide Biosynthesis.....	19
Fate of Glucuronic Acid Conjugates.....	19
UDP-Glucuronyltransferase.....	22
D-Glucuronic Acid Pathway.....	26
Microsomal Oxidation: Relationship to Glucuronidation.....	30
External Factors Affecting Biotransformation Reactions.....	36
Nutritional Influences.....	36
Analytical Methods Used to Quantify Glucuronide Excretion.....	43
Summary.....	53
III. METHODOLOGY.....	56
Subject Recruitment.....	56
Data Collection.....	57
Pre-Experimental Survey.....	58
Food Frequency Questionnaire.....	58
Urine Collection.....	59

Laboratory Analysis.....	60
Urinary Creatinine Determination.....	60
Urinary Conjugated Glucuronic Acid Determination.....	62
Variables to Assess.....	68
Vegetable, Fruit and Meat Consumption.....	68
Dietary Non-Nutrients.....	69
Genetics.....	72
Summary.....	73
IV.    RESULTS.....	74
Subjects.....	74
Conjugated Glucuronic Acid Excretion vs. Various Factors.....	84
Dietary Factors.....	84
Non-Dietary Factors.....	85
Exposure to Chemicals.....	87
Genetics.....	87
Comments.....	88
V.    DISCUSSION.....	91
VI.   SUMMARY AND CONCLUSIONS.....	103
REFERENCES.....	105
APPENDICES.....	114
A. Consent Form.....	115
B. Detoxification Profile Study 1984.....	117
C. Pre-Experimental Survey.....	118
D. Food Frequency Questionnaire.....	125

E. Creatinine.....	129
F. Exposure of Subjects to Chemical Substances.....	131
VITA.....	139
ABSTRACT	

## LIST OF TABLES

Table	Page
1. The eight classical conjugation reactions.....	9
2. Glucuronidation which results in activation of xenobiotics.....	11
3. Range of xenobiotic structures glucuronidated....	24
4. Urinary conjugated glucuronic acid excretion calculated from Fishman and Green.....	44
5. Urinary conjugated glucuronic acid excretion calculated from Mazzuchin et al.....	46
6. Total glucuronic acid concentrations determined in urine samples by the CL and NR methods....	52
7. Urinary excretion of conjugated glucuronic acid for the one-day sample.....	75
8. Between-subject variation in urinary excretion of conjugated glucuronic acid for the one-day sample.....	77
9. Urinary excretion of conjugated glucuronic acid for the three-day sample.....	80
10. Within-subject variation in urinary excretion of conjugated glucuronic acid for the three-day sample.....	81
11. Urinary excretion of conjugated glucuronic acid according to certain factors for the one-day sample.....	86
12. Between-subject variation in urinary excretion of conjugated glucuronic acid for the three-day sample using three- day average.....	89
13. Urinary excretion of conjugated glucuronic acid according to certain factors for the three-day Sample.....	90

## LIST OF FIGURES

Figure	Page
1. Interactions between chemical compounds and the organism.....	6
2. Interactions between MFO and conjugase enzymes...8	8
3. Structure of D-glucuronic acid.....15	15
4. Structure of UDPglucuronic acid.....17	17
5. Synthesis of UDPGA and glucuronidation reactions.....	18
6. Conversion of $\alpha$ -UDPGA to $\beta$ -glucuronide in glucuronidation reaction.....	20
7. Two models proposed as the mechanism for the latency of UDPGT activity.....	27
8. D-glucuronic acid pathway.....	29
9. Proposed scheme for the metabolism of substrates by the cytochrome P-450 -containing monooxygenases.....	32
10. Hypothetical reaction sequence for microsomal drug hydroxylation and glucuronidation.....	33
11. Proposed clusterlike arrangement of the drug-metabolizing enzymes within the microsomal membrane.....	34
12. Glucuronide hydrolysis coupled with lucigenin chemiluminescence.....	51
13. Flow chart for urinary conjugated glucuronic acid determination.....	63
14. Standard curve plot of absorbance units vs. P-Gide concentration.....	65
15. Boxplot representative of between-subject variability of conjugated glucuronic acid (mmole/24 hr) for the one-day sample.....	78
16. Boxplot representative of between-subject variability of conjugated glucuronic acid (mole/mole creatinine) for the one-day sample.....	79

17.	Boxplot representative of between-subject variability (mmole/24 hr) for the three-day sample.....	82
18.	Boxplot representative of within-subject variability (mole/mole creatinine) for the three-day sample.....	83
19.	Summary of the effects of dietary nutrients on drug metabolism.....	96
20.	Side-by-side boxplots representative of the variability in urinary conjugated glucuronic acid excretion (mmole/24 hr) among caffeine users.....	97
21.	Side-by-side boxplots representative of the variability in urinary conjugated glucuronic acid excretion (mole/mole creatinine) among caffeine users.....	98
22.	Summary of the influence of dietary non-nutrients on drug metabolism.....	100
23.	Dynamic interactions among dietary factors that may influence drug response in humans.....	103

## CHAPTER I: INTRODUCTION AND OBJECTIVES

On a daily basis people come in contact with hundreds of chemical substances in an infinite number of combinations. These chemicals reach us by way of food, water, medications, the air, and via skin contact. They are found in domestic, occupational, and recreational settings.

While many of these substances are benign and some are necessary in order to sustain life, some compounds which are present in the diet or elsewhere in the external environment are of no known functional value to us. Such exogenous, nonbeneficial compounds are termed xenobiotics (1). Exposure to xenobiotics is considered undesirable to a normal healthy species as it may prove harmful. Xenobiotics, then, are potential toxins.

Upon absorption into the body, the fate of these nonnutrient compounds varies. Excretion by way of the urine, perspiration, or expired air is possible for those which are water soluble. Lipophilic compounds, which can be excreted in the feces, tend to accumulate in the body. This accumulation may elicit a toxic response (2). To counter this, lipophilic xenobiotics are subject to

enzymatic reactions in the liver which render them more water soluble and easily excreted. This process is called biotransformation.

Williams (3) proposed these enzymatic reactions to be of two types: phase I and phase II reactions. During phase I reactions, foreign compounds are converted to metabolites that can serve as substrates for phase II enzymes. The kinds of reactions which characterize phase I include oxidations, reductions, and hydrolyses. Phase II reactions include acetylations, amino acid conjugations, glucuronidations, glutathione conjugations, methylations, and sulfations.

Products of phase II reactions are called conjugates. Conjugation reactions involve the combination of an endogenous conjugating agent (ex: glutathione, glucuronic acid, sulfur, etc.) with a foreign compound or its metabolite. Conjugation serves to end the biological activity of the compound and is termed a detoxification reaction, although there are recorded examples of metabolic activation resulting from phase II metabolism (4). In general, though, the formation of conjugates plays an essential role in the elimination of xenobiotics from the body.

Formation of glucuronic acid conjugates (glucuronidation) represents the most important phase II reaction (5). Its significance lies in the readily available supply of glucuronic acid in the tissues plus the large number of functional groups which are capable of forming glucuronic acid conjugates (also called glucuronides), the most significant of these being alcohols, phenols, carboxylic acids, thiols, and amines (6).

Conjugation with glucuronic acid can occur both with and without previous phase I metabolism, depending upon the nature of the xenobiotic being conjugated. According to Dutton (7) the balance between the toxification and detoxification of phase I products is delicate and may be upset by genetic changes, age, hormones, diet, and drug treatment. These factors, then, may influence the detoxification of xenobiotics by glucuronidation.

The main objective of this research is to assess the 24 hour urinary excretion of conjugated glucuronic acid in a free-living population of 117 young adult males. This will provide a profile of detoxification via glucuronidation for the population. Additionally,

1. The range and intraindividual variation of

conjugated glucuronic acid excretion over three consecutive days for a subgroup of 40 males will be examined.

2. Information regarding each individual's frequency of exposure to xenobiotics will be examined.
3. Information regarding each individual's dietary habits will be examined.
4. Information obtained from 2 and 3 will be compared to the detoxification profile obtained to determine whether correlations exist between the amount of conjugated glucuronic acid excreted and the xenobiotic and/or dietary information.

## CHAPTER II: REVIEW OF LITERATURE

### 2.1 Conjugation of Xenobiotics

#### 2.1.1 Background Information

When xenobiotic compounds enter the body, several alternatives may occur: (a) the xenobiotic may be excreted unchanged, if the compound is already very polar and unreactive to cellular constituents; (b) the xenobiotic may undergo spontaneous (non-enzymatic) reactions to form new products that may be more or less reactive than the parent compound; and (c) the xenobiotic may undergo enzymatic metabolism to enhance its clearance from the body (usually resulting in the conversion of a lipophilic compound to a more hydrophilic form) (8). The balance between metabolism which results in activation of a compound and metabolism which results in its detoxification determines the degree of toxicity of the compound (Figure 1).

The enzyme systems most responsible for detoxifying xenobiotic compounds are the mixed-function oxidases (MFO) or monooxygenases and several conjugating enzymes including glucuronyltransferase, sulfotransferase, and glutathione transferase. The enzymes are prevalent in the liver,

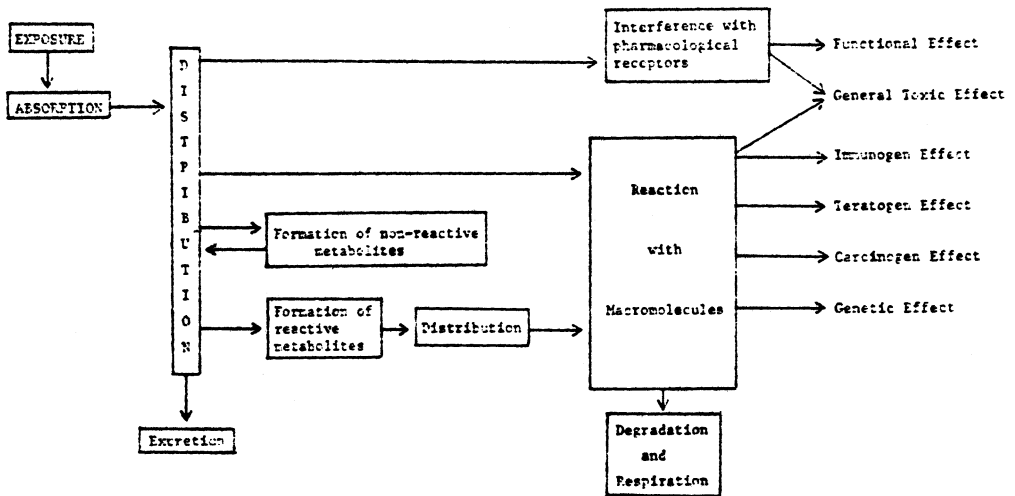


Figure 1: Interactions between chemical compounds and the organism (70).

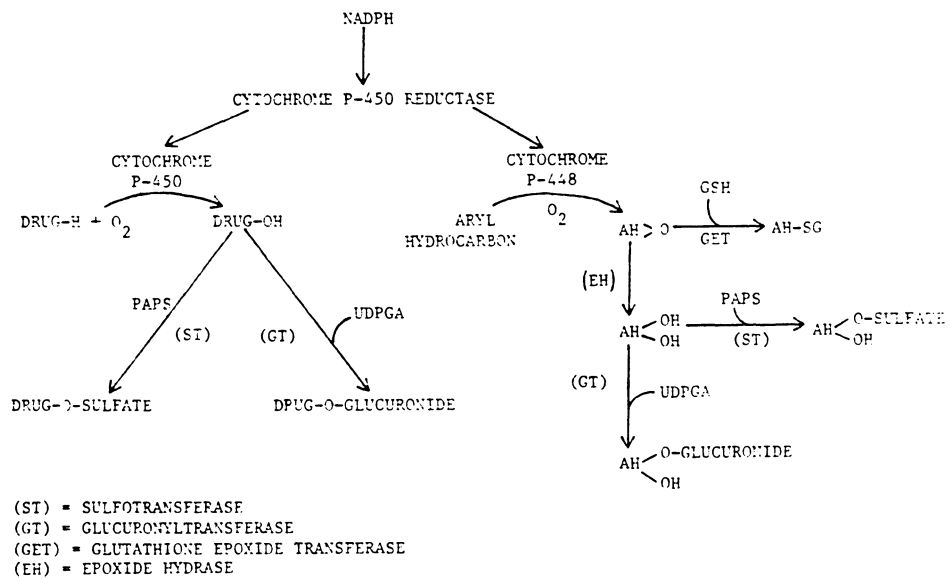
although the small intestine, kidney, lung, and various other tissues also possess a degree of detoxification ability. Interactions between the components of the MFO and conjugase enzymes during xenobiotic detoxification is shown in Figure 2.

The enzymatic reactions which alter existing functional groups or introduce polar groups onto non-polar compounds are the phase I reactions which are directly related to the MFO reactions. The phase II reactions include those that specifically conjugate polar groups of xenobiotic compounds with endogenous cofactors, such as glucuronate, sulfate, and glutathione.

Caldwell (9) defines conjugation as a group of synthetic reactions in which a foreign compound or a metabolite thereof is covalently linked with an endogenous molecule or grouping to give a characteristic product known as a conjugate.

### 2.1.2 The Eight Classical Conjugation Reactions

Table 1 presents the eight major conjugation reactions, all of which involve the combination of a xenobiotic with small endogenous moieties derived from carbohydrate or amino acid sources. Differences occur in terms of the



**Figure 2: Interactions between MFO and conjugase enzymes (52).**

Table 1: The eight classical conjugation reactions (9).

REACTION	CONJUGATING AGENT	FUNCTIONAL GROUPS INVOLVED
Glucuronidation	UDPGA	-OH -COOH -NH <sub>2</sub> -NR <sub>2</sub> -SH >C-H
Glucose conjugation	UDPglucose	-OH -COOH -SH >NH
Sulfation	PAPS	-OH -NH <sub>2</sub> -SH
Methylation	SAMe	-OH -NH <sub>2</sub>
Acetylation	AcetylCoA	-OH -NH <sub>2</sub>
Cyanide Detoxication	sulfane sulfur	CN <sup>-</sup>
Glutathione Conjugation	GSH	arene oxide, epoxide alkyl and aryl halides
Amino Acid Conjugation	gly, gln, orn, taurine	-COOH

nature of the xenobiotic substrate involved, the reaction mechanisms, and the biological consequences.

The products of the majority of the classical conjugation reactions are hydrophilic and are eliminated in the urine and/or bile. Less often, further metabolism of conjugated xenobiotics occurs. Hydrolysis of the conjugate with further phase I and/or phase II reactions is possible (10). In rare instances there may be even further phase I metabolism of conjugates without deconjugation, and even the formation of double conjugates.

Conjugation which results in activation of xenobiotic compounds also is known to occur. Three types of such "active conjugates" may be distinguished, according to Caldwell (9): (a) chemically stable conjugates with biological activity (b) conjugates whose chemical reactivity results in metabolic activation and (c) conjugates which are not end products of metabolism but which undergo further metabolism resulting in biological activity different from that of the parent molecule. Examples involving glucuronidation are given in Table 2.

Organic acids are, in fact, activated by conjugation with glucuronic acid because the carbonyl carbon of the

**Table 2: Glucuronidation which results in metabolic activation of xenobiotics (9).**

GLUCURONIDATION PRODUCING STABLE ACTIVE METABOLITES

compound: morphine  
comment: potent analgesic

GLUCURONIDATION CONTRIBUTING TO FORMATION OF REACTIVE METABOLITES

compound: N-hydroxy-2-acetamido fluorene  
comment: conjugation favors formation of reactive carcinogens

GLUCURONIDATION YIELDING GLUCURONIDES ACTIVE AFTER FURTHER METABOLISM

compound: N-hydroxy-2-acetamido fluorene (N-glucuronide)  
comment: transport form of proximate carcinogen to target (bladder)

ester linkage is susceptible to nucleophilic attack. Acyl glucuronides can react with biological nucleophiles and this may be important toxicologically (11) since acylation of cellular macromolecules by reactive glucuronides is possible. There also have been reports of intramolecular rearrangement by acyl group migration in the ester glucuronides of xenobiotics, although the biological significance of forming various positional isomers of acyl glucuronides is perhaps insignificant (12).

### 2.1.3 Competitive conjugation

When more than one type of conjugation can occur with a compound, the conjugating enzymes (transferases) will compete with each other for the same compound (substrate). Bray et al. (13) have shown that sulfate may become depleted in some instances of substrate overload, in which case competing glucuronidation takes over. The glucuronidation process is known to have a very high substrate capacity and a rate almost as fast as that of sulfation at similar substrate concentrations for certain substances (14).

The rate of disappearance of the substrate and the rates of appearance and disappearance of the conjugates in blood, urine, and bile may indicate whether the rate of

conjugation is affected by either the capacity of one of the conjugating pathways, the rate of supply of the substrate to the site of conjugation, or by blood flow through the organ where conjugation takes place. At increasing substrate concentrations, the conjugating enzymes may become saturated at some point. The concentration of the substrate at the site of conjugation may be very different from that in blood or plasma. The rate at which the substrate is supplied to the site of conjugation may be a factor in competitive conjugation. When conjugation takes place in two or more organs (compartments), differential saturation of the conjugating enzymes involved can occur in each compartment depending upon the pharmacokinetics of substrate entry (15).

Additional factors which influence and determine the mechanism of conjugation reactions with xenobiotics are: species differences, interference by other compounds, and the molecular structure of the functional groups involved in the conjugation (16).

The enzymes involved in drug biotransformation reactions are to a degree regulated by the structure and composition of the membranes to which they are bound. The phospholipid environment of the enzyme

UDP-glucuronyltransferase has been shown to regulate its activity (17). The current views regarding phospholipid-induced regulation of UDP-glucuronyltransferase are outlined in section 2.2.2.

In summary, conjugation reactions involve the combination of an endogenous conjugating agent with a foreign compound or a metabolite, under the influence of a transferase enzyme specific for the conjugating agent. The conjugation reactions are important to the overall response of an organism to foreign compounds, affecting their disposition and elimination. Conjugates in general are acidic, water-soluble compounds, properties which minimize their reabsorption and maximize their excretion. Conjugation of foreign compounds and their metabolites serves to prevent toxic accumulations in an organism, and as such is a life-sustaining process.

## 2.2 The Metabolism of Glucuronic Acid

### 2.2.1 Glucuronic Acid Biosynthesis

In glucuronide biosynthesis, the sugar acid D-glucuronic acid (Figure 3) is coupled with a wide variety of compounds to form glucuronides.

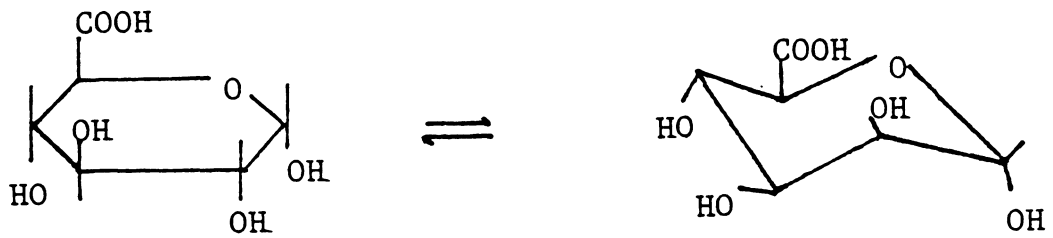


Figure 3: D-glucuronic acid structure.

Dutton and Storey (18) found that glucuronide synthesis required a certain thermostable factor, a compound which was identified as UDPglucuronic acid (Figure 4).

Since the liver was known to contain both UDPglucuronic acid and UDPglucose, it was suspected that UDPglucuronic acid was a metabolite of UDPglucose. Strominger et al. (19) discovered that UDPglucuronic acid was formed in liver tissue from UDPglucose by a specific enzyme, UDPglucose dehydrogenase, and  $\text{NAD}^+$ .

UDPglucuronic acid (UDPGA) is synthesized in a two step reaction, shown in Figure 5. It is not known whether the rate of formation of UDPGA or the transfer of the glucuronic acid moiety to the acceptor substrate is rate limiting in glucuronidation.

UDPGA-synthesizing enzymes are present in many body tissues, and UDPGA synthesis may occur in each of those tissues. Researchers have shown that the rate of synthesis of UDPGA in the healthy liver is sufficient to cope with very high demands. Glucuronidation of harmol in rat liver maintained a high sustained rate for more than one hour (20). Few xenobiotics are known to require such high rates of UDPGA supply for their conjugation, so depletion of

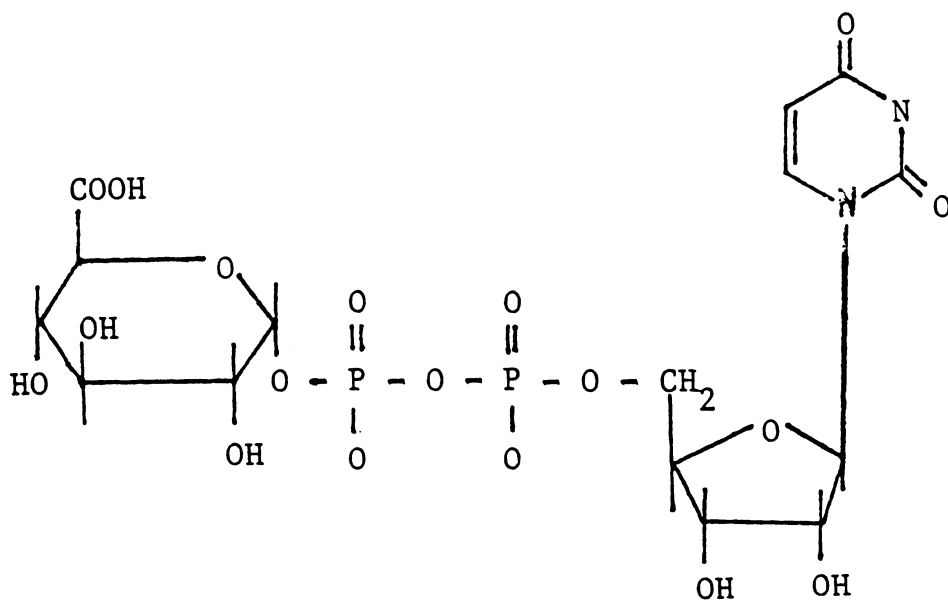


Figure 4: UDPglucuronic acid structure.

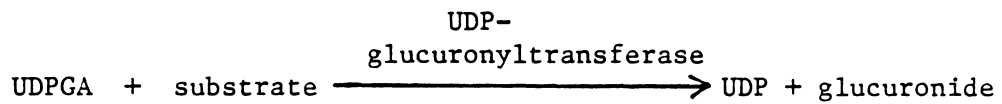
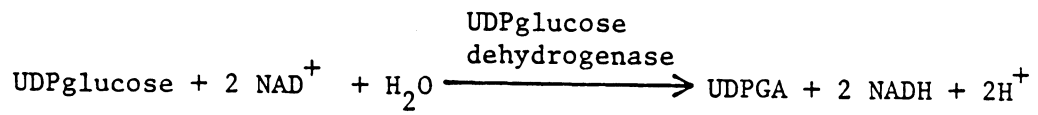
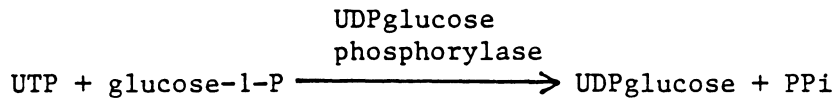


Figure 5: Synthesis of UDPglucuronic acid and glucuronidation reactions.

UDPGA in vivo is an unlikely event.

### 2.2.2 Glucuronide Biosynthesis

In glucuronidation, UDPglucuronic acid (UDPGA) is coupled to a wide variety of compounds to form glucuronides (also called glucuronic acid conjugates, glucosiduronic acid conjugates, or glucopyranosiduronic acid conjugates), as shown in Figure 5.

Axelrod et al. (21) have shown that UDPGA exists in the  $\alpha$ -configuration but inverts to form  $\beta$ -glucuronides (Figure 6) during the reaction catalyzed by UDP-glucuronyltransferase (UDPGT). The glucuronidation reaction seems to involve an attack by the acceptor site or the  $\beta$ -side of the C<sub>1</sub> carbon of glucuronic acid leading to displacement of the UDP from the opposite  $\alpha$ -side.

Transfer of glucuronic acid from UDPGA to a nucleophilic site on a suitable acceptor substrate molecule (called the aglycone) by UDPGT appears to be the only major pathway for glucuronide biosynthesis (22).

### 2.2.3 The Fate of Glucuronic Acid Conjugates

The conjugation of UDPglucuronic acid with various exogenous and endogenous compounds results in the formation of several classes of compounds according to Tomasic (23):

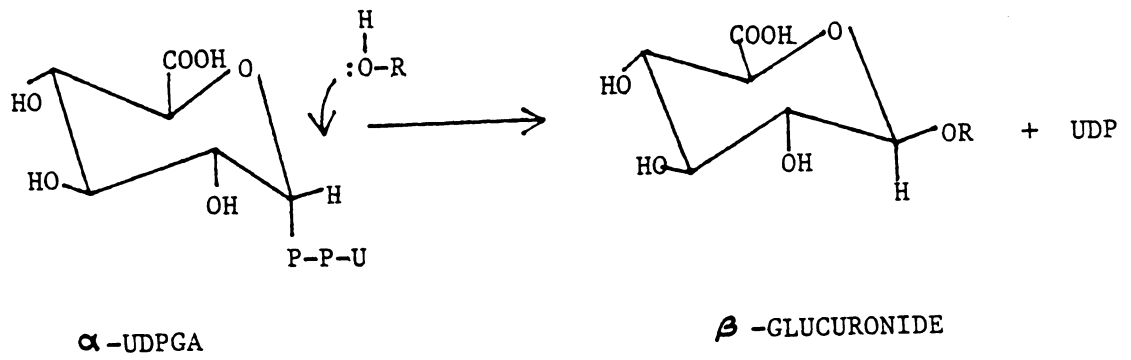


Figure 6: Conversion of  $\alpha$ -UDPGA to  $\beta$ -glucuronide in glucuronidation reaction.

glucuronides (ether, enol, and N-hydroxy), 1-O-acyl glucuronides ("ester"), N-glucuronides, and S-glucuronides.

Ether glucuronides are formed from primary, secondary, and tertiary alcohols and phenols (ex: steroid glucuronides). In enol glucuronides, the aglycone is conjugated with UDPGA through an enolized ketone group. N-hydroxy compounds form N-substituted glucuronides. Compounds containing a carboxyl group form 1-O-acyl glucuronic acids. Compounds containing an aromatic or aliphatic amino group, a sulfonamide or carbamoyl group, or heterocyclic nitrogen atom form N-glucuronides. Compounds containing the sulfhydryl group form S-glucuronides. C-glucuronides are also known to exist, and have been described by Dutton (7) in his review of glucuronidation.

Conjugation of substances (aglycones) with glucuronic acid results in the formation of strongly acidic, ionized compounds which are excreted in the urine and bile. The mechanism by which the body directs some compounds to the bile and some to the urine is not clear. According to Levine (24) compounds with molecular weights greater than 500 are generally excreted in the bile by humans, while those with molecular weights less than 300 are excreted principally in the urine. Compounds of intermediate

molecular weights may be excreted by either route. Hirom et al. (25) have stated that for these intermediate weight compounds, occlusion of one route of excretion results in a compensatory excretion via the other.

The cycle of enterohepatic circulation, wherein the products of biliary excretion are deposited in the duodenum to be either excreted in the feces or reabsorbed from the intestine into the bloodstream, is responsible for the conservation of many substances within the body (ex: the bile salts). In the case of a drug or some other xenobiotic which is converted to its glucuronide and then undergoes enterohepatic circulation, further metabolism of the conjugate can occur. This is due to the presence of the bacterial flora within the large bowel. These microorganisms possess a glucuronidase enzyme which is capable of hydrolyzing the conjugate and releasing the free parent compound which can then be reabsorbed. Any conclusions regarding the fate of xenobiotics which undergo enterohepatic circulation must be carefully considered.

#### 2.2.4 UDP-Glucuronyltransferase (UDPGT)

The UDP-glucuronyltransferases are a family of enzymes of unknown number which are able to catalyze the transfer

of UDPglucuronic acid (UDPGA) to an acceptor compound. These enzymes catalyze the glucuronidation of both endogenous compounds (ex: bilirubin and steroid hormones) as well as exogenous xenobiotic compounds. Many xenobiotics which are not themselves substrates can be metabolized to compounds that are substrates via phase I metabolism (sections 2.1.1 and 2.2.6)

Table 3 lists the functional groups known to be glucuronidated, and shows the structures for substrates in each class.

UDPGT activity has been detected in most body tissues, including the adrenal gland, brain, diaphragm, gastrointestinal mucosa, heart muscle, kidney, liver, lung, skin, spleen, thymus, and testis. The liver, due to its high specific activity and large size, plays the major role in the total glucuronidating capacity of the organism. Glucuronidation in other tissues is important only in instances when the tissue is a port of entry of the xenobiotic to the body (lungs, skin) or when the substance to be glucuronidated is generated or accumulated in the tissue (26).

It is possible to increase the activity of UDPGT in tissues by treatment with certain substances. This

**Table 3: Range of xenobiotic structures glucuronidated (43).**

GROUP	STRUCTURE
Linkage through oxygen	
Aryl-OH	Ar · O · GA
Aryl or alkyl enolic	$\begin{array}{c}   \\ \text{CH}=\text{C} \cdot \text{O} \cdot \text{GA} \end{array}$
Alkyl-OH (1, 2, 3)	$\begin{array}{c}   \\ -\text{C} \cdot \text{O} \cdot \text{GA} \\   \end{array}$
Acyl-OH (aryl or alkyl)	-COO · GA
Hydroxylaminic	$\begin{array}{c} -\text{N} \cdot \text{O} \cdot \text{GA} \\   \end{array}$
Linkage through sulfur	
Thiolic	-S · GA
Carbodithiolic	-C · S · S · GA
Linkage through nitrogen	
Amino	AR · NH · GA
Ureido	-NH · CO · NH · GA
1-Thioureido	NH · CS · NH · GA
Sulfonimido	SO <sub>2</sub> · N · GA
Heterocyclic	=N · GA
Linkage through Carbon	$\begin{array}{c} \diagdown \\ -\text{C} \cdot \text{GA} \end{array}$

enhancement is called induction and is thought to be a consequence of increased de-novo synthesis of UDPGT. Although inducibility appears to be a general property of UDPGT, the degree of induction varies according to tissue and inducing agent. It also is postulated that differences in the microenvironment of UDPGT in liver vs. extrahepatic tissues accounts for at least some of the differences in induction/activation of the enzyme (26).

Species differences in UDPGT activities are known. Among mammals, the cat possesses negligible UDP-glucuronyltransferase activity with the most common exogenous aglycones, while the guinea pig has the highest activity for a wide range of substrates (27).

Latency, or the observed delay in the activity of an enzyme, is displayed by various integral microsomal enzymes including UDPGT. Two models have been proposed as the mechanism for latency of the activity of UDPGT. The first, referred to as the "conformational model" (28), imagines that each UDPGT can exist as different conformational forms or isomers, being stabilized differentially by the nature of the phospholipid in their environments. This sort of conformational constraint by the membrane microenvironment is postulated to maintain UDPGT in a catalytically

incompetent conformation. The presence of a suitable allosteric effector or activator is then required to "switch on" the enzyme (29).

The "compartmentation model" for latency proposes that the active sites of UDPGT(s) are located on the inside of the microsomal vesicle, which is not freely permeable to UDPGA (29), meaning that access of substrate to the enzyme is limited. The latency of the enzyme activity is thought to reflect a removal of the barrier to permeability which would thus allow free access of substrates to active sites (Figure 7).

This simple compartmentalization model has been modified in order to account for the base-line activity of the enzyme in intact, untreated microsomal vesicles and for its activation by the compound UDP-N-acetylglucosamine. In the modified model, a specific transport protein for UDPGA is envisioned, one whose function is activated by UDP-N-acetylglucosamine (30).

#### 2.2.5 The D-Glucuronic Acid Pathway

UDPGA is also an intermediate in the D-glucuronic acid pathway, which leads to the synthesis of further metabolites of UDPGA. In a sequence of biochemical reactions, hexose is transformed via UDPglucose,

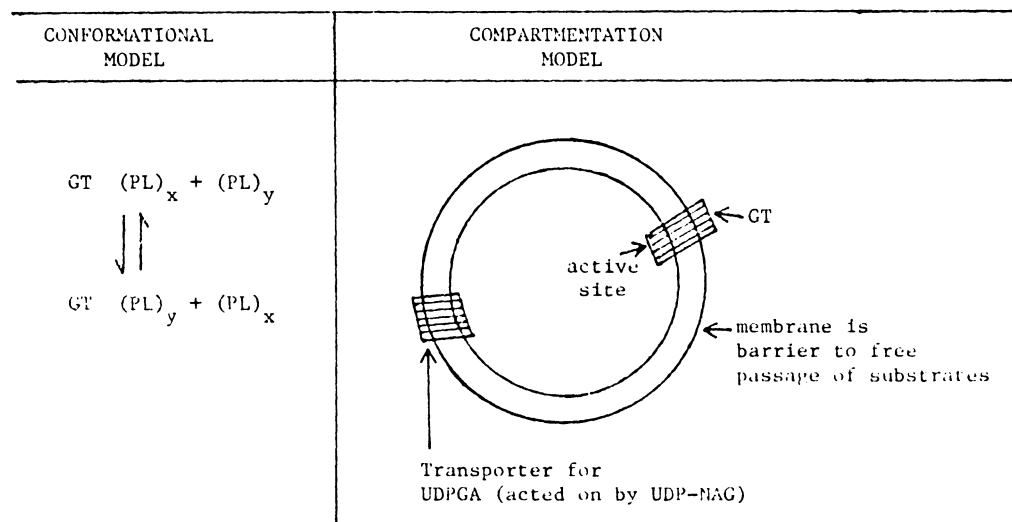


Figure 7: Two models proposed as the mechanism for the latency of UDPGT (43).

UDPglucuronic acid, and D-glucuronic acid to L-ascorbic acid, D-xylulose, and D-glucaric acid. This overall process is referred to as the glucuronic acid pathway (Figure 8).

The metabolism of glucuronic acid via xylulose into the pentose phosphate cycle (hexose monophosphate shunt) represents an additional route for the catabolism of glucose.

Further metabolism of glucuronic acid is associated with the detoxification process, according to Conney (31), who noticed that drugs which stimulate the xenobiotic metabolizing enzymes of the microsomes also stimulated the glucuronic acid pathway without themselves being necessarily conjugated with glucuronic acid.

Notten and Henderson (32) reported stimulation of the glucuronic acid pathway in rats eight hours after exposure to sodium barbital and four other exogenous compounds, without concomitant increases in glucuronidation. Ethanol administration resulted in enhanced glucuronide excretion but without stimulation of the glucuronic acid pathway, which is demonstrated by increased urinary excretion of end products of the cycle (ex: gulonic, ascorbic, and glucaric acids). Therefore, these investigators concluded that

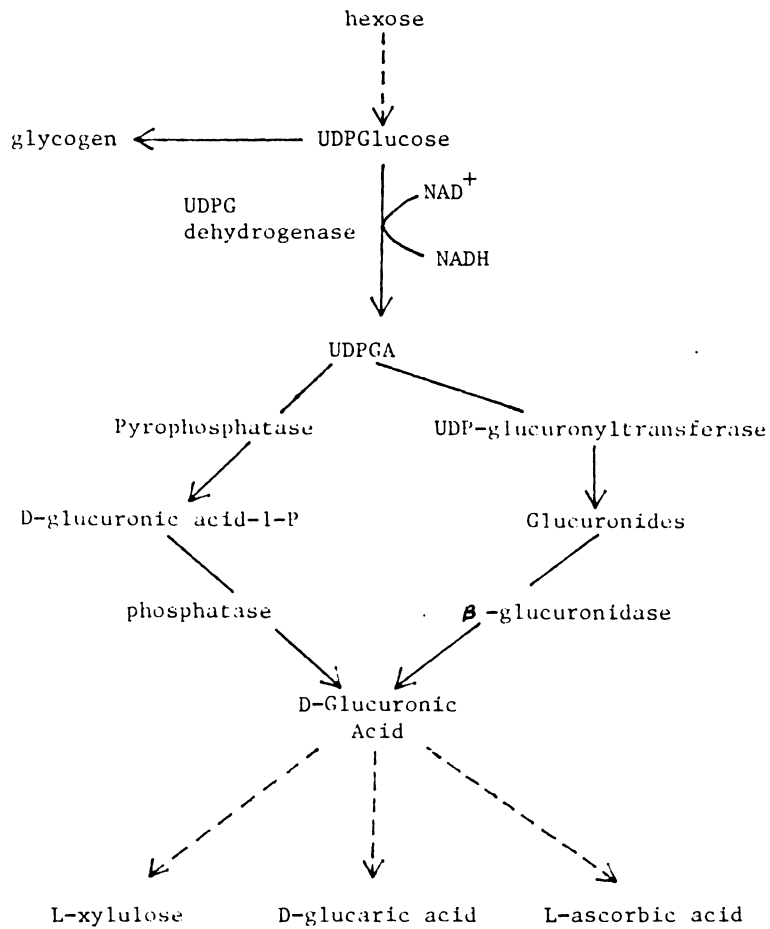


Figure 8: The D-glucuronic acid pathway (33).

stimulation of the glucuronic acid pathway is not directly related to accelerated glucuronidation. They also have stated that stimulation of the glucuronic acid pathway is based upon increased availability of UDP glucose in the liver cell due to an inhibition of glycogen synthesis, and is independent of enhanced UDPGT activity (33). The authors suggested using the urinary D-glucaric acid excretion as a reliable non-specific test for diagnosing exposure to chemical compounds as well as the drug-metabolic capacity.

#### 2.2.6 Microsomal Oxidation: Relationship to Glucuronidation

The biotransformation of xenobiotics in the liver is accomplished by the enzyme systems which reside in the endoplasmic reticulum of hepatocytes. Both rough and smooth endoplasmic reticulum function to assemble the enzymatic complexes which transform foreign compounds, and also serve as the site of those transformations (34). Kidney, lung, intestine, brain, and skin also contain measurable activities of these enzyme systems, which is conventionally classified according to Mason (35) as the mixed function oxidases (MFO).

MFO are comprised of cytochrome P-450, a

heme-containing enzyme, and NADPH-cytochrome c reductase, which is also known as NADPH-cytochrome P-450 reductase. The MFO, or cytochrome P-450-containing monooxygenases, are able to catalyze the introduction of oxygen atoms into xenobiotics of widely different structure (Figure 9).

UDP-glucuronyltransferase, the enzyme which catalyzes the glucuronidation reaction, is situated in close proximity to the microsomal vesicles, and is thought to be localized in the inside compartment (36). Figure 10 represents a hypothetical reaction sequence for microsomal oxidation of a substrate followed by glucuronidation.

The above sequence is consistent with the fact that many compounds undergo glucuronidation following hydroxylation by MFO. Furthermore, since the two reactions occur in the same subcellular structure, it has been assumed that they are functionally and topochemically closely related.

Vainio (37) has speculated that the microsomal mixed function oxidase and UDP-glucuronyltransferase systems may form a vectorially oriented multienzyme complex in the microsomal membrane (Figure 11).

Both systems have been increased *in vivo* following administration of various chemicals (38,39) with induction

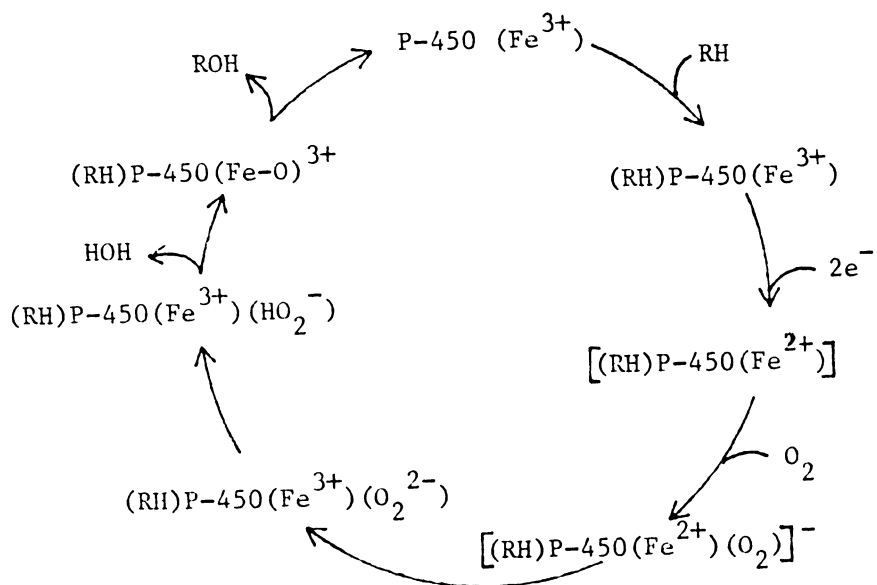


Figure 9: Proposed scheme for the metabolism of substrates by the cytochrome p-450-containing monooxygenases (2).

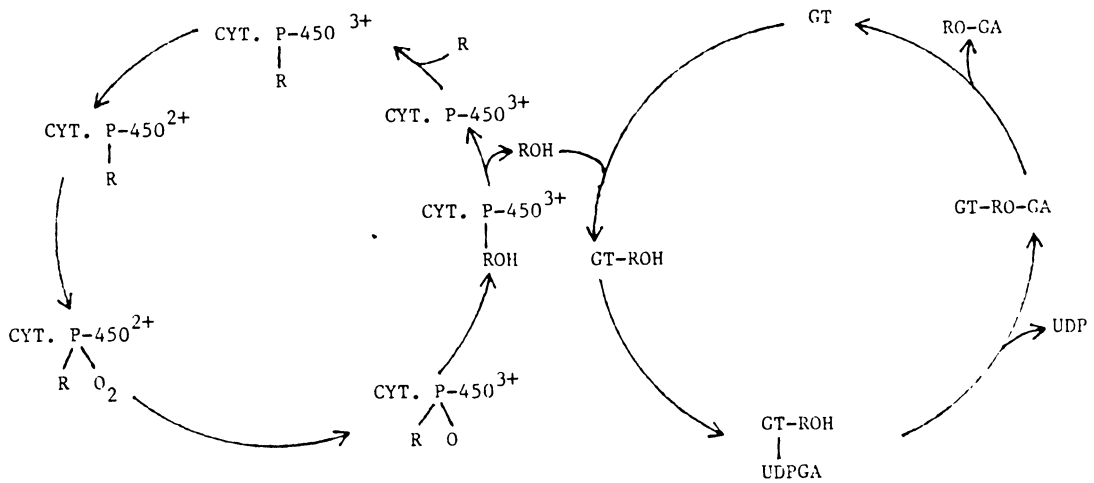


Figure 10: Hypothetical reaction sequence for microsomal drug hydroxylation and glucuronidation (37).

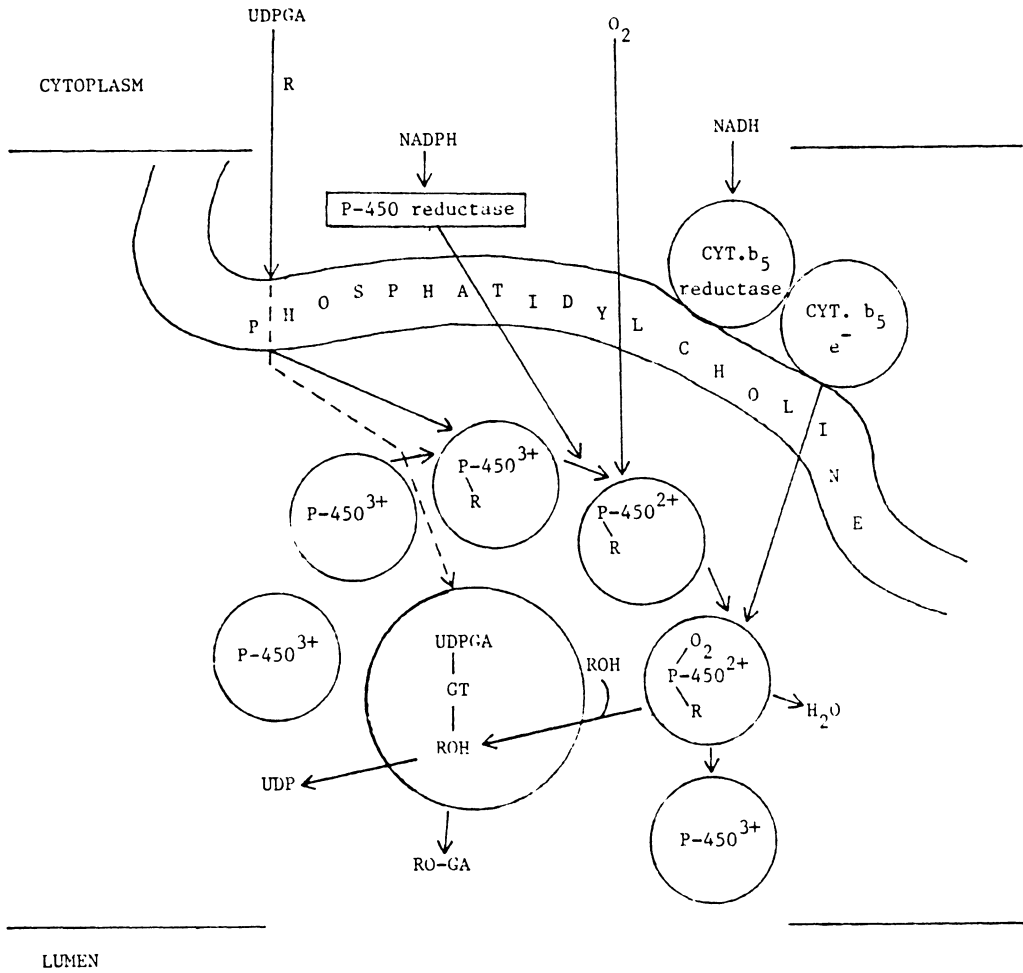


Figure 11: Proposed clusterlike arrangement of the drug-metabolizing enzymes within the microsomal membrane (37).

in the hydroxylation step preceeding that in the glucuronidation step.

Recently, Ehrich and co-workers (40) noticed that microsomes prepared from the livers of chicks given enzyme inducers ( $\beta$ -naphthoflavone and 3-methylcholanthrene) had an increased capability to convert aflatoxin P<sub>1</sub> to its glucuronic acid conjugate. Increased glucuronidation was attributed to increased specific activity of the microsomal enzymes.

It has been reported that aflatoxin B<sub>1</sub> will inhibit enzymes responsible for the metabolism of foreign compounds, including itself (41). Ehrich's group determined that glucuronidation of aflatoxin P<sub>1</sub> was inhibited by 48% in chicks fed aflatoxin B<sub>1</sub> for 40 days, further demonstrating the coupled nature of the MFO and glucuronidation.

Genetic studies in mice have revealed that a common receptor regulates the concentration of UDP-glucuronyltransferase and cytochrome P-450-dependent benzo(a)pyrene hydroxylase (42). Since increases in activities of cytochrome P-450 are due to actual induction (increased mRNA leading to increased enzyme synthesis), a coordinated regulation of increases in the activity of

UDP-glucuronyltransferase and cytochrome P-450 may constitute evidence, according to Vessey and Zakim (43), for the idea that the increased activity of UDP-glucuronyltransferase after administration of cytochrome P-450 inducers is due to true induction of UDP-glucuronyltransferase.

## 2.3 External Factors Affecting Biotransformation Reactions

### 2.3.1 Nutritional Influences

The effects of specific dietary alterations on drug metabolism in humans has been studied only within the last decade. Drug oxidations mediated by cytochrome P-450 have received the greatest attention while conjugations have been less studied. In many experiments, drugs which are extensively oxidized in the liver, such as antipyrine, theophylline, and phenacetin, were administered to fasting individuals. The relationship between the diets the subjects had been receiving and the half-lives of the drugs was therefore able to be examined without the complication of drug-nutrient interactions.

Alvares et al. (44) fed six healthy male subjects a high protein diet (44 percent of total calories) for two weeks followed by a high (70 percent of total calories)

carbohydrate diet for two weeks. The study entailed an isocaloric substitution of carbohydrate for protein; dietary fat remained at roughly 20 percent of total calories. The mean plasma half lives of antipyrine and theophylline (given at day 10 and 14 of each dietary period) were almost 50 percent shorter during the high protein intake than during the high carbohydrate intake. The drugs were therefore metabolized more rapidly during the period when subjects were ingesting the high protein diet.

The opposite effect was seen, however, in a study wherein either a 100g supplement of sodium caseinate (protein supplement) or a 200g sucrose supplement (carbohydrate supplement) were fed to four subjects consuming a well-balanced diet (15 percent protein, 50 percent carbohydrate, 35 percent fat). The well-balanced control diet was fed for two weeks prior to addition of the supplements to their regimens. Removal rates of antipyrine and theophylline from plasma were increased with the sucrose-supplemented diet rather than the casein-supplemented diet (45).

Anderson and co-workers (46) studied the effects of substituting fat for carbohydrate or protein. Six normal

males were sequentially fed high (80%) carbohydrate, high (70%) fat, and high (50%) protein diets over a total study period of six weeks. Each diet was fed for two weeks and antipyrine and theophylline metabolism was examined on days 10 and 14 of each sequence. The mean half-lives for the two drugs were found to be longer during the high carbohydrate as well as high fat dietary periods than during the high protein period. The researchers concluded that a substitution of dietary protein for either carbohydrate or fat was shown to accelerate drug metabolism, whereas substituting fat for carbohydrate had little or no effect.

During protein restriction in rats, activity of UDP-glucuronyltransferase has been seen to increase. A seven day protein-deficient diet was associated with a 100 percent increase in UDPGT activity in rat liver towards two substrates (p-nitrophenol and o-aminophenol) (47).

Anderson and associates (46) found that adding saturated fat (butter) or polyunsaturated (corn oil) fat in place of carbohydrate in the diet had no apparent influence on the metabolism of antipyrine and theophylline. These findings are in contrast to those from animal studies, where the amounts of saturated and unsaturated fatty acids

in the diet can influence the mixed function oxidase system. Mucklow et al. (48) confirmed that exchanging saturated for unsaturated fat in the diet had no significant effect on drug oxidations in humans.

The mechanisms of diet-induced changes in drug metabolism rates have not clearly been established. Phospholipid may be involved in molecular associations between cytochrome P-450 and the flavoprotein reductase, in electron transfer to cytochrome P-450, and may provide binding sites for substrates (49). The diet influences the amounts of phospholipid in the liver endoplasmic reticulum and their fatty acid compositions as well (50). The addition of cholesterol to olive oil or cocoa butter diets caused a significant elevation in hepatic UDP-glucuronyltransferase activity in rats (51). It was the contention of these authors that the activities of the drug-metabolizing enzymes was dependent upon the structure of the membrane, which in turn, was altered by the cholesterol content of the dietary lipid.

The integrity of the microsomal membrane has been shown to be essential for proper functioning of the MFO. The membrane is approximately 35 percent lipid, of which 85 percent are phospholipid. The composition of the membrane

is alterable by variations in the dietary lipids; without the essential fatty acids present in the diet, microsomal membrane lipids do become more saturated. As said, the few studies examining the effects of unsaturated versus saturated fats fed to humans have shown no major changes in MFO activities. This may be due to the time duration of the dietary changes, the composition of the existing body fat, and the limited number of substrates evaluated (52).

Vegetables contain a variety of natural substances which may affect oxidation and conjugation reactions. Rats fed commercial laboratory stock diets containing alfalfa as well as diets containing Brussels sprouts, cabbage, and other vegetables have shown increased oxidative xenobiotic metabolism in the small intestine and liver (53). Certain indoles have been observed to be potent inducers of MFO enzymes in the liver and intestine (54), as has safrole (55), a plant constituent used as a flavoring agent, and some of the flavonoids.

Pantuck et al. (56) studied the effects of feeding a diet containing Brussels sprouts and cabbage for a seven day period to ten normal individuals. A small but significant (13 percent) decrease in the mean antipyrine plasma half-life and an increase (11 percent) in the mean

metabolic clearance rate indicated a stimulatory effect of these vegetables on the metabolism of this drug. Another drug administered to those receiving the cabbage-Brussels sprouts diet, phenacetin, was shown to undergo enhanced oxidative metabolism to N-acetyl-p-aminophenol (APAP). The mean ratio of conjugated APAP to unconjugated APAP in plasma increased, suggesting that the feeding of these vegetables could also enhance glucuronic acid conjugation of this metabolite.

Charcoal-broiling of meats results in the formation of polycyclic aromatic hydrocarbons similar to those found in cigarette smoke (57). Since the oxidative metabolism of several compounds, including antipyrine, theophylline, caffeine, and phenacetin is known to be accelerated in smokers, presumably due to the presence of the polycyclic hydrocarbons, it has been suggested that feeding charcoal-broiled beef to normal subjects might achieve the same effect. A study on antipyrine, phenacetin, and theophylline metabolism in normal subjects who received charbroiled meats twice daily was performed by Kappas and associates (58). O-dealkylation of phenacetin was enhanced, and the mean half-lives of antipyrine and theophylline were decreased. These results indicate that

charcoal broiling of meats can enhance the oxidation rates of certain substrates in humans. The authors commented that glucuronic acid conjugation of APAP is not increased by charcoal-broiled feeding, in contrast to the effect seen with cabbage-Brussels sprouts consumption.

In a rat study, alcohol lowered UDPGA levels in more than 60 percent of the rodents after 72 hours (59). Glucuronidation of acetaminophen, increased by fasting alone, was decreased in the presence of alcohol. The investigators reasoned that the metabolism of alcohol, which increased the intracellular NADH/NAD ratio, would decrease the conversion of UDPglucose to UDPglucuronic acid and thereby diminish the UDPGA levels available for glucuronidation.

The situation of drug metabolism in humans during starvation is complex. Some reports indicate no change in drug clearance in fasted obese patients (60) while children with protein-energy malnutrition exhibited impaired drug oxidations and conjugations (61). The latter example may indicate a multifactorial mechanism for the effects seen, however, as severe protein-energy malnutrition is regularly accompanied by several nutritional deficiencies as well as fatty infiltration of the liver.

#### 2.4 Analytical Methods Used to Quantify Glucuronide Excretion

Direct estimation of glucuronic acid conjugates in biological material has been accomplished by several means. Methods based upon reaction of naphthoresorcinol and carbazole with glucuronic acid have been used with success for colorimetric determination of the glucuronic acid equivalent in the glucuronide moiety.

Fishman and Green (62) established a procedure for determining glucuronides in the presence of glucuronic acid based upon the oxidation of glucuronic acid and other interfering aldehydes with alkaline hypiodite solution. The remaining glucuronide, being protected from oxidation by its linkage to the aglycone, was analyzed by the naphthoresorcinol color reaction. The strongly acid conditions employed in the naphthoresorcinol reaction cleaved the glycosidic linkage, and liberated the glucuronic acid from its aglycone. This gave rise to a blue-violet pigment which was extracted into an organic solvent (toluene) and measured photocolometrically at 560 to 570 nm. Urinary glucuronides were determined as mg per 24 hr (Table 4).

Table 4: Urinary conjugated glucuronic acid excretion calculated from (62).

---

<u>Subject</u>	<u>Conjugated Glucuronic Acid (mmoles/24 hr)</u>
1	1.67, 1.83, 1.86
2	2.06, 2.32, 1.99, 2.38

---

The use of alkaline conditions for the oxidation limits the application of this method to alkali-stable ether glucuronides. Fishman and Yuki (63) successfully used the carbazole reaction of Dische (64) who reacted hexuronic acid with carbazole instead of naphthoresorcinol in the differential analysis of free glucuronic acid, glucosiduronic acid, and hyaluronic acid.

One problem with the above methods when analyzing physiological fluids is that of glucose interference. Mazzuchin et al. (65) reported a modification of the naphthoresorcinol method wherein alkaline degradation of free glucuronic acid using sodium hydroxide was used. Glucose was selectively oxidized by glucose oxidase prior to the assay of the conjugates. The method was examined extensively for accuracy and reproducibility. Up to an eight-fold increase in sensitivity compared to previous naphthoresorcinol procedures was claimed. Glucuronic acid assays were determined from 5 to 100 ug with the best results in the 5 to 50 ug range (average error 5%). Urinary conjugated glucuronic acid was determined as mg/24 hr (Table 5).

Table 5: Urinary conjugated glucuronic acid excretion calculated from (65).

---

<u>Sample</u>	<u>Conjugated Glucuronic Acid (mmoles/24 hr)</u>
1	1.61
2	2.06
3	2.52

---

The use of NaOH for the degradation of free glucuronic acid limited the applicability of the procedure to ether glucuronides.

The use of methods based upon the naphthoresorcinol and carbazole reaction allows for the quantitative assay of glucuronic acid conjugates in physiological fluids. The disadvantages they share, however, are the inability to distinguish between the individual metabolites, and the absence of information regarding the nature of the aglycone.

Use of the lead salt technique, in which glucuronides are precipitated as their lead salts, was reported by Kamil et al. (66). In this method, saturated normal lead acetate was added to urine at pH 4. The first precipitate was discarded and the supernate brought to pH 8. Saturated aqueous basic lead acetate was then added in excess. Lead was removed by treatment of the resulting precipitate with H<sub>2</sub>S yielding the glucuronide alone in the supernate. An advantage to this method is that the mild conditions permit isolation of both acid- and alkali-labile glucuronic acid conjugates. Unfortunately, the overall recovery of the glucuronide is usually poor.

Paper and thin-layer chromatography techniques have

been employed in the analysis of glucuronic acid conjugates. These conjugates, following separation, can be eluted from the paper strips and characterized.

Alkali-labile "ester" glucuronides (1-O-acylglucuronic acids) have been detected in the urine based upon their ability to form characteristic hydroxamic acids after reacting with hydroxylamine. Since free glucuronic acid and conjugates with nonacyl groups do not yield hydroxamic acids, a method for identifying, purifying, or estimating ester glucuronides directly was possible. Schachter (67) presented a technique for the estimation of acyl glucuronides based upon the hydroxylamine reaction. Stable hydroximates of benzoate, salicylate, and probenecid were recovered from the urine of a male subject. These hydroxamate derivatives were formed at neutral pH and at room temperature after a two hour incubation of the patient's urine with freshly prepared hydroxylamine. Identification of the specific metabolites was accomplished by extraction with ether and subsequent use of paper chromatography. A single hydroxamate spot was detected for each compound using Whatman No. 1 paper and n-butanol:glacial acetic acid as the solvent system. Each spot was identified as a specific metabolite based upon

comparison of Rf values with those of reference compounds.

Wagner and Hollmann (68) have described an enzymatic method for the quantitative determination of conjugated glucuronic acid. The technique is based upon the NAD-linked oxidation of uronic acids to the corresponding dicarboxylic acids and is measured in the presence of uronic acid dehydrogenase. In a multi-step procedure, urine samples were sequentially incubated with dehydrogenase,  $\beta$ -glucuronidase, and then dehydrogenase enzymes. The initial oxidation of free glucuronic acid in the sample yielded a corresponding NADH concentration. The  $\beta$ -glucuronidase enzyme was then used to cleave off glucuronic acid from its aglycone. This produced additional free glucuronic acid in the sample, which was then re-oxidized with the dehydrogenase enzyme and another determination of NADH concentration was made. The amount of conjugated glucuronic in the original sample was therefore equivalent to the difference in initial and final NADH concentrations.

Determination of conjugated glucuronic acid by combining enzymatic hydrolysis with lucigenin chemiluminescence has been performed recently by Klopf and Nieman (69). Lucigenin (N,N-dimethyl-9,9-diacridinium

nitrate) is known to undergo chemiluminescent (CL) reaction in alkaline solution in the presence of reductants (ex: glucuronic acid) and oxygen.

The technique involves passing the glucuronide-containing urine through a small column (IMER, or immobilized enzyme reactor) packed with  $\beta$ -glucuronidase enzyme. The glucuronide bond is hydrolyzed and the glucuronic acid concentration can be determined by the lucigenin CL reaction (Figure 12).

Table 6 presents a comparison of total glucuronic acid concentrations determined in urine samples by the CL method vs. the NR (naphthoresorcinol) method. The higher values obtained by the NR method were thought to be due to "the effects of other species on the NR reagent" (69).

The presence of competing organic reductants in urine would interfere in a determination of glucuronic acid by this method. Chromatographic separation of glucuronic acid from the other reductants was reported to be successful in eliminating this interference (69).

In the above discussion, the methods available to directly assay and quantify glucuronides in biological fluids were presented. Separate from these are techniques used to isolate glucuronic acid conjugates from biological

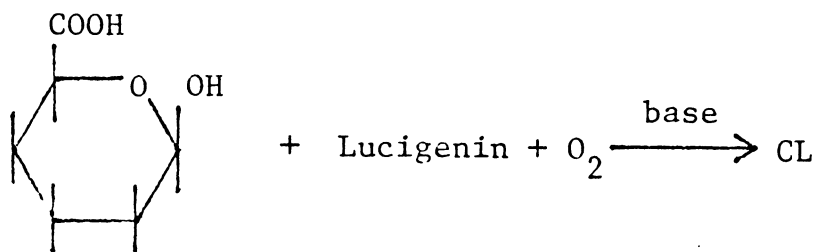
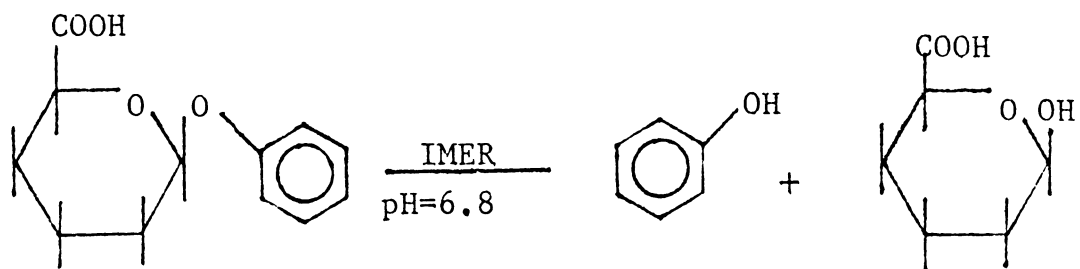


Figure 12: Glucuronide hydrolysis coupled with lucigenin chemiluminescence (CL) (69).

Table 6: Total glucuronic acid concentrations determined in urine samples by the chemiluminescence (CL) and naphthoresorcinol (NR) methods (69).

SAMPLE	mM glucuronic acid	
	CL METHOD	NR METHOD
1	1.59	2.16
2	1.14	2.03
3	2.32	3.62
4	0.47	1.85
5	3.09	4.53
6	2.28	2.88
7	0.41	1.47
8	1.72	3.27

fluids, including: adsorption onto activated charcoal, adsorption onto XAD resins, use of Sephadex gel filtration, and use of ion-exchange chromatography (23). These are nonspecific processes which yield complex mixtures that require further separation; therefore their use as a means to quantify glucuronides is limited. Such procedures are useful however as a basis for further analysis.

A great body of data has accumulated in recent years regarding the separation and characterization of specific glucuronic acid conjugates. The techniques involved in this area of glucuronide analysis include: counter-current distribution, high resolution liquid chromatography, gas chromatography, mass spectrophotometry, nuclear magnetic spectroscopy, and infrared spectroscopy (23).

Other research has focused upon the pharmacokinetic aspect of drug conjugations. Also, clinical investigations have attempted to correlate the concentration of conjugates in biological fluids to various diseases. Both Tomasic (23) and Dutton (7) have summarized the work in these areas.

## 2.5 Summary

Glucuronidation is one of several phase II detoxification reactions which compete for various

endogenous and exogenous substrates. It is quantitatively the most important and can occur in all mammalian tissue but especially in the liver. The substrate required for glucuronide formation, UDPglucuronic acid, is part of intermediary metabolism and closely related to glycogen synthesis. The enzymes involved in the formation of UDPGA are located in the cytosol.

As the chemical environment in which we live has become increasingly complex owing in large part to the growing array of both intentional (i.e.: medicines) and incidental (i.e.: environmental pollutants) xenobiotics, interest in glucuronidation reactions has expanded apace. Many factors are known to affect the rate and pathway of xenobiotic metabolism, with diet and the environment representing external influences while age, sex, hormones, and disease represent internal influencing factors.

Given the importance of glucuronidation and the interest in conjugation reactions, relatively little has been reported in the scientific literature regarding the normal total daily excretion of conjugated glucuronic acid in humans. By contrast, much effort has been put forth in quantifying the excretion of specific, characterized glucuronides in pharmacokinetic experiments.

The main objective of this study was to quantify the urinary conjugated glucuronic acid excreted by adult male humans representative of the "free-living" population at large. As such, a broad spectrum of environmental differences, genetic differences, and dietary habits was represented. As these factors are thought to influence xenobiotic disposition (71), dietary and environmental data was obtained from questionnaires completed by the subjects who submitted urines for analysis. A secondary objective of this study was to examine what association, if any, several factors (dietary, environmental, and genetic) might have on the excretion of glucuronic acid conjugates in these subjects.

## CHAPTER III. METHODOLOGY

### 3.1 Subject Recruitment

Healthy adult males who were not taking medications and who were not under a physician's care, were invited to participate in the Human Detoxification Profile Study. The subjects were recruited through advertisements in the campus paper and through the distribution of posters and fliers around campus and the town of Blacksburg. A cash compensation of \$10.00 was offered to each subject as an incentive to participate. The advertisement campaign was conducted until approximately 150-200 subjects were recruited.

Weekly meetings were scheduled for the purpose of explaining the objectives of the Human Detoxification Profile Study to all persons interested. Additionally, the rights and responsibilities of the subject, the possible risks and benefits of participation, and the detailed procedures for data collection were carefully explained as required by the Institutional Review Board for Research With Human Subjects. At the closure of each meeting, all volunteers who had decided to participate were asked to sign the Informed Consent Form which is presented in

Appendix A. Personal information was also obtained for contacting purposes, such as the scheduling of the data collection period. All persons who were interested in the study, but unable to attend an introductory meeting, were given a handout "Detoxification Profile Study 1984" which is presented in Appendix B, along with an individual explanation.

### 3.2 Data Collection

Considering the limitations of time, manpower, and collection bottles, a maximum of 45 subjects were scheduled each week; therefore, a total period of 4-6 weeks were required for sample and questionnaire collection. During the collection period, each subject was allowed to pursue a normal unrestricted lifestyle with one exception. The only imposition on the environment of the subject was to abstain from the use of medications for three days prior to and during the collection period. It was emphasized that if the use of medications became necessary, the subject was then expected to report the identity and dosage of the drug, as well as the frequency and duration of consumption. This emphasis was an attempt to encourage honesty and cooperation since the data from subjects

consuming medications during the three-day collection period were excluded.

### 3.2.1. Pre-Experimental Survey

Each subject was required to complete the Pre-Experimental Survey, which is presented in Appendix C, and to submit it prior to the scheduled urine collection period. The Survey was an attempt to quantitate the frequency of exposure to caffeine, alcohol, tobacco, social drugs, medications, and environmental xenobiotics. It also provided a means of describing the subject population. To ensure anonymity, each subject was assigned a code number for use on all data that was collected. Hopefully, this enhanced the honesty and reliability of answers to delicate questions in the Survey, such as the use of social drugs.

### 3.2.2. Food Frequency Questionnaire

During the collection period, no impositions were placed on the regular dietary habits of the subjects, except that weight maintenance was expected. Subjects who were involved in a weight loss or weight gain program were excluded from participation in the study. Each subject was required to complete the Food Frequency Questionnaire which is presented in the Appendix D. The purposes of the

questionnaire were to obtain presumptive evidence of dietary adequacy and to obtain descriptive information on the dietary patterns of the population. The foods which were presented in the questionnaire were categorized according to the ADA Exchange Plan (72). Considering the limitations of accuracy, reliability, and time requirements involved in the collection of detailed dietary records (73, 74), and considering the type of nutrition information required by the Human Detoxification Profile Study, the development of a Food Frequency Questionnaire was considered to be most appropriate, least expensive, and easiest to administer for a large population.

### 3.2.3. Urine Collection

On the Monday of the scheduled collection period, each subject was given two acid-washed and autoclaved collection bottles (polypropylene, 1 liter) which were clearly labelled with the subject code number and collection date. The handout, "Instructions for Collection and Handling of Urine" which is included in the Appendix, was distributed to each subject along with an oral explanation of the procedures. The urine was collected on Tuesday, Wednesday, and Thursday, as three consecutive, yet individual 24-hour periods. 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. Hence, on Wednesday, Thursday, and Friday mornings after the first voiding of urine, each 24-hour specimen was submitted to the laboratory and new collection bottles were distributed. Additional collection bottles were readily available for subjects requiring more than 2 liters in a 24-hour period.

Upon the delivery of each 24-hour specimen, the weight of each subject was recorded, and questions were asked concerning any deviations from the collection and handling procedures; specifically, incomplete collections or spillages, and consumption of medications. Using acid-washed volumetric cylinders, the total volume of each specimen was recorded, and after thorough mixing, pH determinations were made. Several aliquots from each specimen were obtained and immediately frozen for future analysis.

### 3.3 Laboratory Analysis

#### 3.3.1. Urinary Creatinine Determination

The Technicon Auto Analyzer (Technicon Instrument Corp., Tarrytown, N.Y.) was employed for the determination

of urinary creatinine. 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 reagents required for the automated creatinine technique were prepared according to the handout, "creatinine" which is presented in Appendix E and was provided by the Technicon Instruments Corp., N.Y. The handout also contains a diagram and a general description of the operation of the Auto Analyzer. The standards were prepared from a Fisher Scientific Creatinine Stock Solution of 1 mg/ml in 0.1M HCl.

Randomly selected, frozen urine samples which had been aliquoted for creatinine determination were thawed in the refrigerator for 24 hours, and then placed in a warm water bath to accelerate the achievement of room temperature prior to analysis. Duplicate 1 ml samples of urine were diluted to 5 mls with deionized water and placed in a sample tray of the Auto Analyzer following a series of standard samples. A graphic recorder was attached to the colorimeter for recording the peak absorbance values of the standard and urine samples. Less than 5% error between duplicate samples was accepted and the average urinary creatinine concentration was reported as mg creatinine/kg

body wt/24 hr. The intraindividual and interindividual variability in creatinine excretion/kg body wt/24 hr was determined for the population and each sample exceeding 2 standard deviations from the average within and among variability were excluded from further analysis.

### 3.3.2 Urinary Conjugated Glucuronic Acid Determination

The modified naphthoresorcinol (MNR) method of Mazzuchin et al. (65) was used in the quantitation of urinary conjugated glucuronic acid for this project. A flow chart for this procedure is given in Figure 13.

This method was chosen on the basis of its ability to:

- (a) prevent glucose and free glucuronic acid, both of which are NR-positive substances, from interfering in the assay of conjugated glucuronic acid
- (b) exhibit improved sensitivity at low levels of conjugated glucuronic acid when compared to previous methods with a molar absorptivity of  $30.1 \times 10^3$
- (c) provide a more stable reagent (MNR reagent) than was available with previous methods
- (d) provide a technique applicable to quantifying conjugated glucuronic acid present in human urine at the optimum concentration range with error

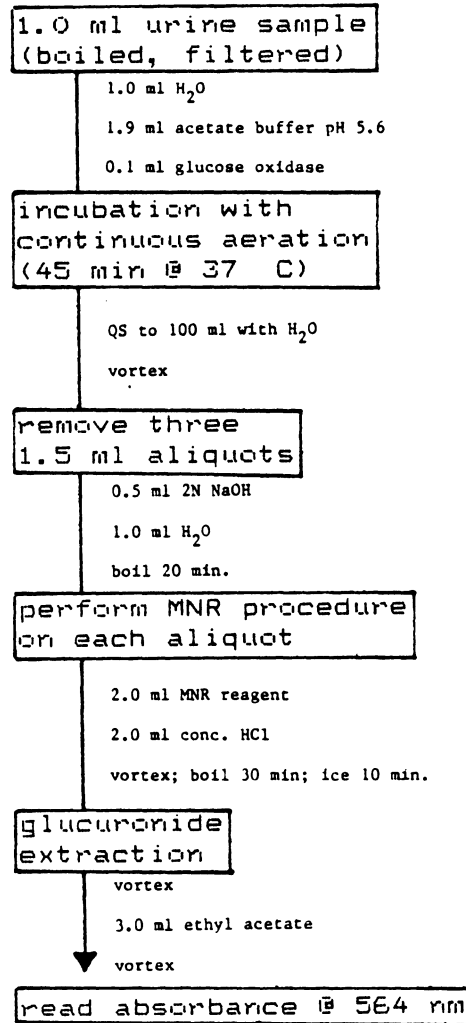


Figure 13: Flow chart for urinary conjugated glucuronic acid determination.

limits claimed at +/- 5% .

Glucose interference is selectively eliminated with glucose oxidase enzyme. This permits the determination of conjugated glucuronic acid though the urine may contain high glucose concentrations.

In the determination of conjugated glucuronic acid, the free glucuronic acid present in urine must be eliminated. Sodium hydroxide is used to decompose free glucuronic acid to a product insensitive to the NR reagent.

The method uses an naphthoresorcinol reagent (NR) prepared as follows: 400 mg NR is dissolved in 80 ml distilled, deionized water and 0.5N sodium hydroxide is added dropwise to adjust the pH between 8 - 8.5. After 15 minutes, 10% (w/v) phosphoric acid is added from a pasteur pipet until the pH reaches between 2 - 2.5. Sodium bisulfite (200 mg) is added and the volume is brought up to 100 ml with distilled water. The solution is then filtered twice, wrapped in a foil-covered flask, and refrigerated until use. The reagent will remain stable for seven days. The total time required for a complete analysis is approximately four hours. This analysis was limited to quantification of alkali-stable ether glucuronides. No attempt was made to distinguish between the individual

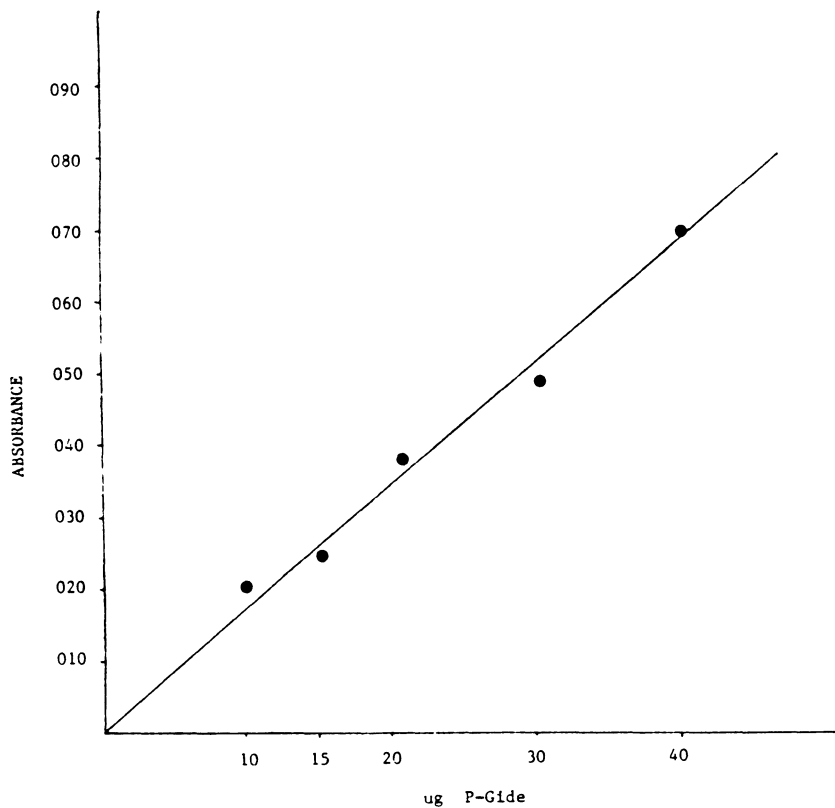


Figure 14: Standard Curve Plot of Absorbance vs. P-Gide (phenolphthalein glucuronide) Concentration. Linearity of the assay for P-Gide standard added to human urine. 1.0 ml of solutions containing 0, 10, 15, 20, 30, and 40 ug of P-Gide standard were added to 1.0 ml of diluted human urine and subjected to the entire MNR procedure for the assay of conjugated glucuronic acid in the urine. Each point represents the average of three determinations ( $r = 0.997$ ).

metabolites or to identify the aglycones.

Before determining the urinary conjugated glucuronic acid excretion for the subjects in this study, a standard curve of diluted urine spiked with a known concentration of phenolphthalein glucuronide (P-Gide) was prepared.

The standard curve (Figure 14) was obtained for spike concentrations of 0 - 40 ug/ml, with a linear increase in absorbance vs. P-Gide concentration. Recoveries of P-Gide added to the urine assay system were over 85% in the concentration range examined, indicating good sensitivity. There was an interassay variation of 12.9% for six determinations.

For the urinary conjugated glucuronic acid determination, one day was randomly selected and analyzed for each of 117 subjects that complied with the collection protocol, compliance being based upon a creatinine excretion within two standard deviations from the mean (mg creatinine/24 hrs/kg body weight). The group of data which passed this criterion was the sample from which the random selections were made. Also, all three days for a randomly selected subgroup of forty subjects were analyzed for determining the within-subject variability.

The actual determination of urinary conjugated

glucuronic acid excretion was according to the following method (Figure 13). A well-mixed 5.0 ml aliquot of urine was filtered through Whatman No. 541 filter paper, boiled for 20 minutes in a 100 C water bath, and filtered again to remove any precipitate. For the conjugated glucuronic acid analysis (Figure 13), 1.0 ml of this boiled and filtered urine was then incubated at 37 C for 45 minutes along with 1.9 ml acetate buffer, pH=5.6 and 0.1 ml glucose oxidase type V in a 100-ml volumetric flask. Continuous aeration was achieved by blowing air into the flask from the laboratory hood air valve using a pasteur pipet attached to a manifold connected to the air line. After the incubation period, the flasks were brought up to volume with distilled, de-ionized water. The flasks were inverted to mix the contents, and three 1.5 ml aliquots of diluted urine were removed and transferred to test tubes containing 0.5 ml 2N NaOH and 1.0 ml of distilled, de-ionized water. These samples were then boiled for 20 minutes. After this time, to the samples were added 2.0 ml MNR reagent and 2.0 ml concentrated HCl. The test tubes with sample were then vortexed, boiled for 30 minutes, and then put in ice for 10 minutes. The test tubes were again vortexed, and 3.0 ml ethyl acetate was added to each tube which was re-vortexed.

The acetate layer was pipetted into a quartz cuvette and the absorbance was read against a blank at 564 nm in a Bausch & Lomb Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N. Y.). Concentrations of conjugated glucuronic acid were determined from the standard curve and corrected for the dilution factor. The standard curve measured concentrations of P-Gide. The concentration of conjugated glucuronic acid alone was determined by multiplying the P-Gide concentration by the ratio of the molecular weights of the glucuronic acid moiety to that of P-Gide.

### 3.4 Variable to Assess

#### 3.4.1 Vegetable, Fruit, and Meat Consumption

Data obtained from the food frequency questionnaire were used to ascertain the frequency of consumption of various foods and also charbroiled foods. Frequencies of vegetable consumption, meat consumption, and charbroiled food consumption were analyzed and subjects were categorized as being either low consumers, moderate consumers, or high consumers as follows:

Low Range (L) = 0 - 3 times/month

Moderate Range (M) = up to once/day

High Range (H) = more than once/day

Meat intake was chosen as a potential variable affecting conjugated glucuronic acid excretion because meat is a good source of protein and high protein intake has been shown to enhance drug metabolism (44,46).

Vegetable intake was chosen as a potential variable affecting conjugated glucuronic acid excretion because diets high in certain vegetables have been associated with an increase in drug metabolism and enhanced glucuronidation (56).

Fruit intake was chosen as a potential variable affecting conjugated glucuronic acid excretion because fruit is a good source of carbohydrate and feeding high carbohydrate diets has been shown to enhance drug clearance in humans (45). Additionally, UDPglucuronic acid is formed from the simplest unit of carbohydrate, glucose.

#### 3.4.2 Dietary Non-Nutrients

Charbroiled meat intake was chosen as a potential variable affecting conjugated glucuronic acid excretion because increased consumption of these meats can increase oxidative enzyme activity (58). Both benzo(a)pyrene, a polycyclic hydrocarbon and inducer of cytochrome P-450, and

tryptophan pyrolysis products, which are compounds derived from the amino acid tryptophan and known to induce cytochrome P-448, are found in charbroiled foods. Subjects were categorized as being either low (L) or high (H) consumers as follows:

Low Range (L) = 0 - 3 times/month

High Range (H) = > 3 times/month

Data obtained from the pre-experimental survey was used to ascertain each subject's exposure to various substances. Tobacco smoking was chosen as a potential variable affecting conjugated glucuronic acid excretion because increased tobacco use can enhance drug metabolism (75). Tobacco users were classified as follows:

Low (L) = smoking less than 1 cigarette/day

Moderate (M) = smoking 1-10 cigarettes/day

High (H) = smoking more than 10 cigarettes/day

Alcohol use was chosen as a potential variable affecting conjugated glucuronic acid excretion because alcohol is known to inhibit the synthesis of UDP-glucuronic acid from UDP-glucose by increasing the intracellular NADH/NAD ratio (76). Alcohol users were classified as follows according to their consumption of alcoholic beverages:

Low (L) = 3 times/month or less (including non-users)

Moderate (M) = up to 6 times/week

High (H) = up to 3 times/day or more

Marijuana was chosen as a potential variable affecting conjugated glucuronic acid excretion because marijuana smoke contains high levels of polycyclic hydrocarbons as well as delta-tetrahydrocannabinol compounds which may stimulate drug metabolizing enzymes. Marijuana users were classified according to use as follows:

Low (L) = less than once/week (including non-users)

Moderate (M) = 1 to 4 times/week

High (H) = greater than 4 times/week

Chemical exposure was chosen as a potential variable affecting conjugated glucuronic acid excretion since it is known that numerous chemical substances, including heavy metals, industrial pollutants, insecticides, and the like are potent inducers as well as inhibitors of xenobiotic-metabolizing enzymes. Subjects were first classified as being low, low/medium, medium, high, or very highly exposed (Appendix), based upon their responses to question F of the pre-experimental survey. They were then reclassified into low (low only), moderately (low/medium, medium) or highly

(high, very high) exposed to chemicals.

Caffeine intake was chosen as a potential variable affecting conjugated glucuronic acid excretion since it has been shown that, amounts commonly present in a couple of cups of coffee or tea are equivalent to the pharmacologically active dose, which is a potent inducer of the MFO enzymes (78). Caffeine use was reported as follows:

Low (L) = up to 2 cups/week

Moderate (M) = up to 6 cups/week

High (H) = a cup a day and more

### 3.4.3 Genetics

The intervariability of xenobiotic metabolism is based upon many factors, including genetics (76). Therefore, genetics was chosen as a potential variable affecting conjugated glucuronic acid excretion. In this study, genetic factors were assessed solely in terms of cancer incidences among family members. Subjects who responded "yes" to knowing of cancer incidences among their relatives were classified as "Y" and those having no known relatives with cancer were classified as "N".

#### 3.4.4 Summary

Variables chosen as being potential influences on the urinary conjugated glucuronic acid excretion for the subjects of this study included vegetable, fruit, and meat consumption; consumption of charbroiled food; use of tobacco, alcohol, marijuana, and caffeine; exposure to chemicals, and incidence of cancer in genetically-related individuals.

Since this study did not impose a measured quantity of any of the above variables on the subjects but instead merely obtained intake or exposure frequency data regarding each variable (except genetics) according to the recollection of each subject, it would be unfair to elevate the results of this portion of the study to a "treatment effect of variable x on conjugated glucuronic acid excretion" when in fact none existed. Instead, the mean and standard deviation of urinary conjugated glucuronic acid excreted by each grouping according to the above variables were calculated, and the statistical F-test was employed in testing the hypothesis of no difference between the respective groups.

## CHAPTER IV. RESULTS

### 4.1 Subjects

The 117 subjects used in this study were derived from a group of 135 males, mean age 20.7 years. Dietary analysis indicated the subjects' food intake to be nutritionally adequate (79). Alcohol, tobacco, and caffeine consumption varied, as detailed in section 4.2.2. Their three-day urinary creatinine excretions were measured and analyzed for between-subject and within-subject variability. Creatinine excretion normally remains relatively constant for an individual from day to day. For this study, subjects whose creatinine excretions were at the extremes of the range (greater than two standard deviations from the mean, which was 24.55 mg/kg of body weight/24 hr) and/or whose day-to-day creatinine excretions were inconsistent, were excluded from further analysis.

The mean, standard deviation, and range for the urinary excretion of conjugated glucuronic acid in mmole per 24 hr and in mole conjugated glucuronic acid per mole creatinine per 24 hr for the one-day sample are listed in Table 7. The between-subject variation in urinary conjugated glucuronic acid excretion in mmole/24 hr and mole/mole creatinine/24 hr for the one-day sample is given in Table

Table 7: Urinary Excretion of Conjugated Glucuronic Acid For The One-Day Sample.

<u>Conjugated Glucuronic Acid</u>	<u>Excretion</u>	
	<u>Mean (SD)</u>	<u>Range</u>
mmole/24 hr	0.725 (0.341)	0.228-1.976
mole/mole creatinine	0.0492 (0.0292)	0.0138-0.1981

n = 117

8. Finally, the boxplots of mmole conjugated glucuronic acid per 24 hr and mole conjugated glucuronic acid per mole creatinine per 24 hr for the one-day sample are shown in Figures 15 and 16, respectively.

A three-day urine sample from forty subjects was randomly selected from the 117 subjects whose creatinine excretions were assessed to exhibit acceptable between-subject and within-subject variability. The mean, standard deviation, and range for the urinary excretion of conjugated glucuronic acid for the three-day sample in mmole per 24 hr and in mole per mole creatinine per 24 hr are listed in Table 9. The within-subject variation in urinary conjugated glucuronic acid excretion for the three-day sample in mmole conjugated glucuronic acid per 24 hr and mole per mole creatinine per 24 hr is shown in Table 10. Finally, the boxplots of mmole conjugated glucuronic acid excreted per 24 hr and mole/mole creatinine per 24 hr for the three-day sample, are presented in Figures 17 and 18, respectively.

Table 8: Between-Subject Variation in Urinary Excretion of Conjugated Glucuronic Acid For The One-Day Sample.

<u>Conjugated Glucuronic Acid</u>	Variation <sup>a</sup>	
	<u>Inter</u>	<u>CV<sup>b</sup></u>
mmole/24 hr	0.3410	47.1
mole/mole creatinine	0.0292	59.2

n = 117

<sup>a</sup>Inter-variation reported as standard deviation.

<sup>b</sup>CV = coefficient of variation (%).

78

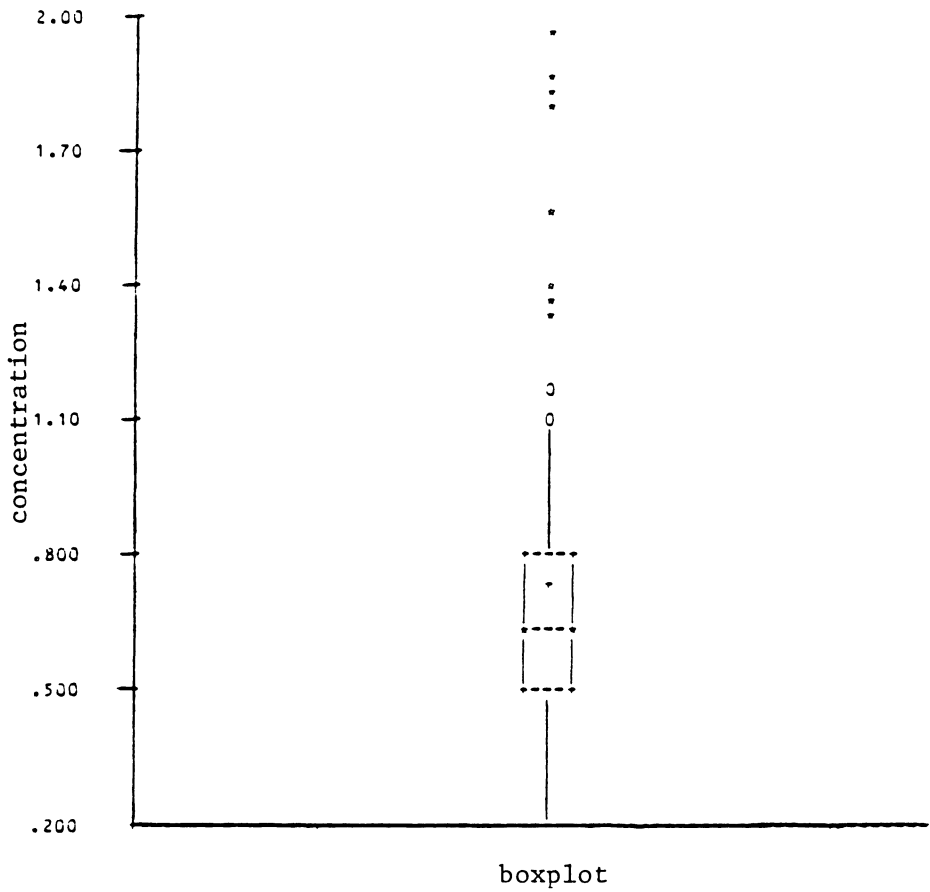


Figure 15: Boxplot representative of between-subject variability of conjugated glucuronic acid (mmole/24 hr) for the one-day sample.

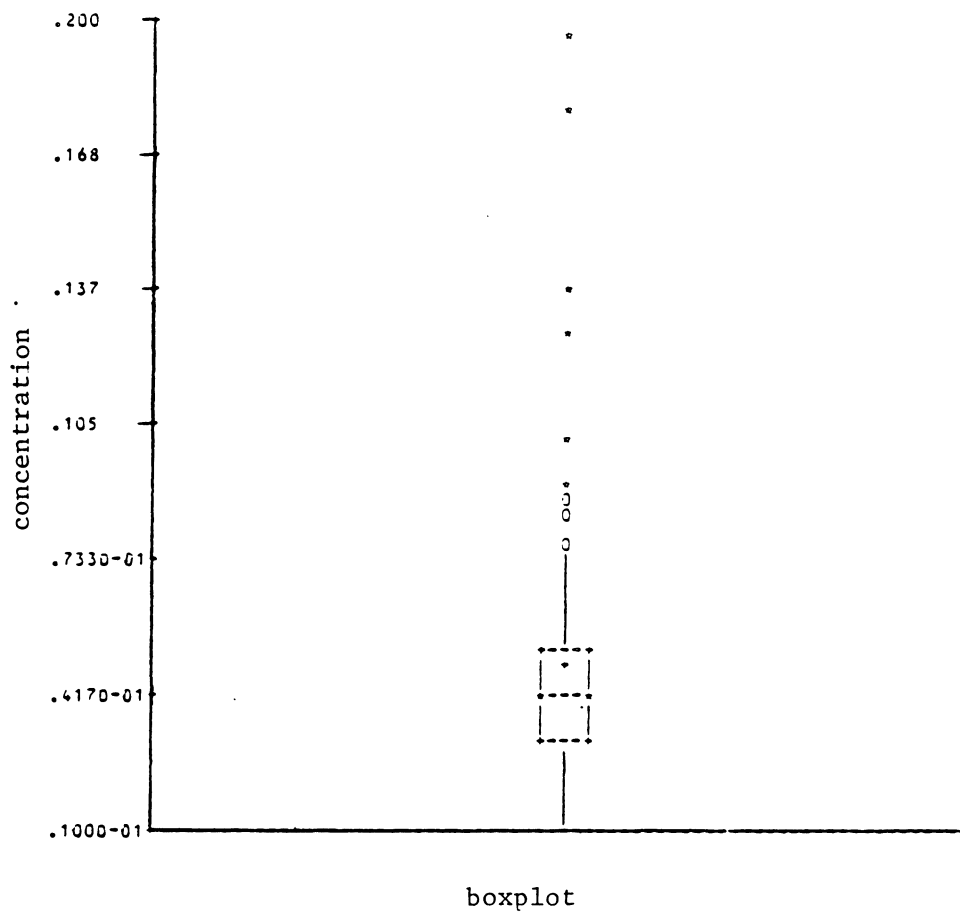


Figure 16: Boxplot representative of between-subject variability of conjugated glucuronic acid (mole /mole creatinine) for the one-day sample.

Table 9: Urinary Excretion of Conjugated Glucuronic Acid For The Three-Day Sample.

---

<u>Conjugated Glucuronic Acid</u>	Excretion	
	<u>Mean (SD)</u>	<u>Range</u>
mmole/24 hr	0.848 (0.409)	0.199-1.893
mole/mole creatinine	0.0562 (0.0294)	0.0138-1.1772

---

n=40

Table 10: Within-Subject Variation in Urinary Excretion of Conjugated Glucuronic Acid For The Three-Day Sample

<u>Conjugated Glucuronic Acid</u>	Variation <sup>a</sup>	
	<u>Intra</u>	<u>CV<sup>b</sup></u>
mmole/24 hr	0.2476	29.2
mole/mole creatinine	0.0161	28.7

n = 40

<sup>a</sup>Intra-variation reported as standard deviation.

<sup>b</sup>CV = coefficient of variation (%).

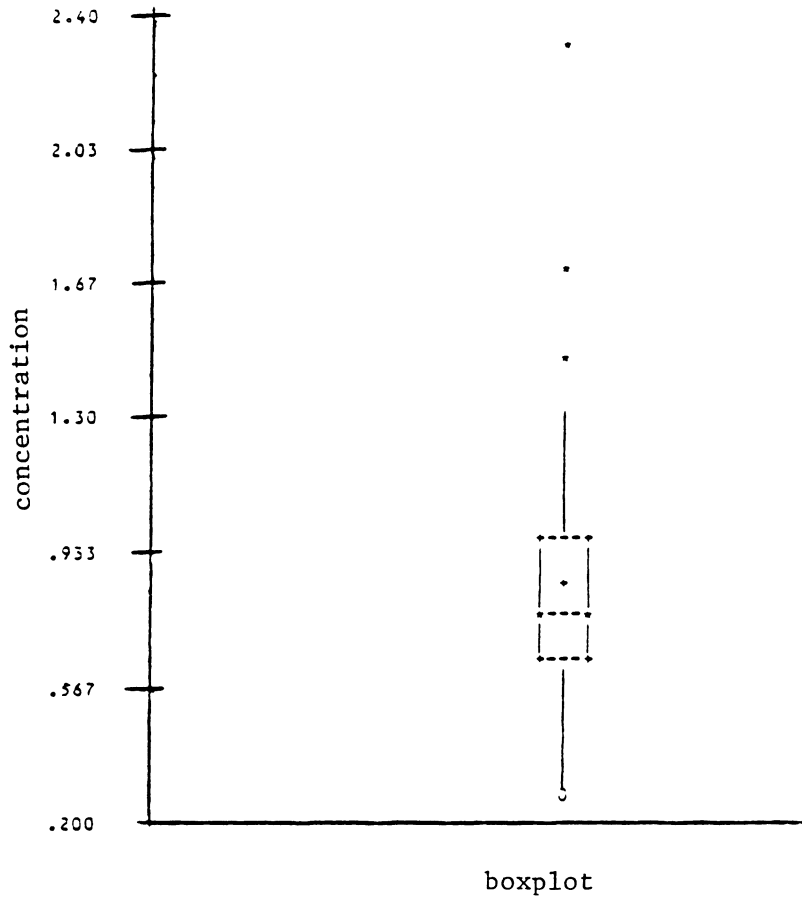


Figure 17: Boxplot representative of within-subject variability (mmole/24 hr) for the three-day sample.

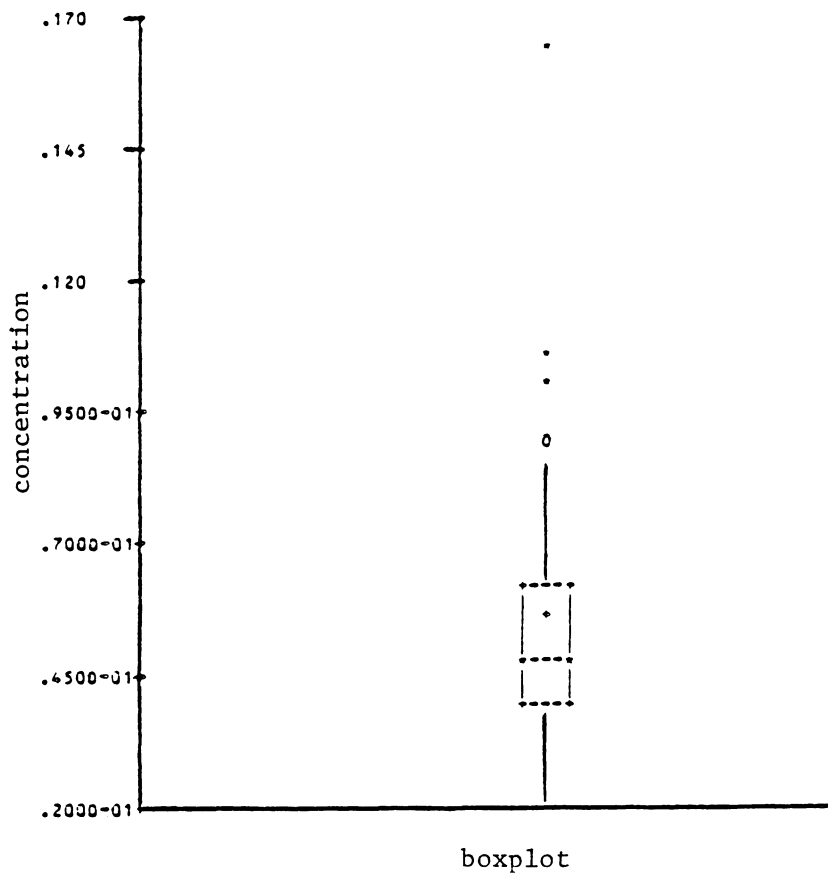


Figure 18: Boxplot representative of within subject variability (mole conjugated glucuronic acid/mole creatinine) for the three-day sample.

## 4.2 Conjugated Glucuronic Acid Excretion vs. Various Factors

### 4.2.1 Dietary Factors

Subjects were classified according to their intake of vegetables, fruit, meat, and charbroiled food as being low, moderate, or high consumers.

For the one-day sample, regarding vegetable consumption, 55% were classified as low, 27% were classified as moderate, and 18% were classified as high.

Regarding fruit consumption, 76% were classified as low, 18% were classified as moderate, and 6% were classified as high.

Regarding meat consumption, 49% were classified as low, 38% were classified as moderate, and 13% were classified as high.

Regarding charbroiled food consumption, 72% were classified as low, and 28% were classified as high.

The mean and standard deviation for the urinary excretion of conjugated glucuronic acid in mmole per 24 hr and mole per mole creatinine per 24 hr for the one-day sample, according to vegetable, fruit, meat, and charbroiled food intake are listed in Table 11.

#### 4.2.2 Non-Dietary Factors

Subjects were classified according to their using tobacco, alcohol, marijuana, and caffeine as being low, moderate, or high users.

For the one-day sample regarding tobacco use, 87% were classified as low, 7% were classified as moderate, and 6% were classified as high.

Regarding alcohol use, 67% were classified as low, and 33% were classified as high.

Regarding marijuana use, 88% were classified as low, 8% were classified as moderate, and 4% were classified as high.

Regarding caffeine use, 54% were classified as low, 20% were classified as moderate, and 26% were classified as high.

The mean and standard deviation for the urinary excretion of conjugated glucuronic acid in mmole per 24 hr and mole per mole creatinine per 24 hr for the one-day sample according to tobacco, alcohol, marijuana, and caffeine use are listed in Table 11.

Table 11: Urinary Excretion of Conjugated Glucuronic Acid According to Certain Factors For The One-Day Sample.

Parameter		n	(mmole/24 hr) <sup>a</sup>	(mole/mole Creat/24 hr) <sup>a</sup>
vegetable intake	L	65	0.718 (0.346)	0.0504 (0.0247)
	M	31	0.778 (0.345)	0.0490 (0.0333)
	H	21	0.667 (0.325)	0.0461 (0.0361)
fruit intake	L	89	0.711 (0.322)	0.0464 (0.0218)
	M	21	0.736 (0.356)	0.0561 (0.0432)
	H	7	0.860 (0.527)	0.0645 (0.0518)
meat intake	L	57	0.7168 (0.354)	0.0501 (0.0278)
	M	45	0.7366 (0.313)	0.0471 (0.0234)
	H	15	0.7190 (0.393)	0.0524 (0.0469)
char-broiled intake	L	84	0.717 (0.355)	0.0499 (0.0312)
	H	33	0.745 (0.308)	0.0475 (0.0236)
tobacco use	L	102	0.741 (0.356)	0.0490 (0.0292)
	M	8	0.577 (0.134)	0.0382 (0.0104)
	H	7	0.654 (0.237)	0.0650 (0.0388)
alcohol use <sup>b</sup>	L	76	0.729 (0.361)	0.0492 (0.0310)
	M	37	0.711 (0.312)	0.0495 (0.0256)
marijuana use <sup>b</sup>	L	100	0.723 (0.342)	0.0499 (0.0305)
	M	9	0.630 (0.208)	0.0421 (0.0180)
	H	4	0.940 (0.603) <sup>d</sup>	0.0503 (0.0140)
caffeine use <sup>c</sup>	L	59	0.685 (0.292)	0.0475 (0.0272)
	M	22	0.646 (0.344)	0.0472 (0.0366)
	H	28	0.845 (0.436) <sup>d</sup>	0.0547 (0.0285)
chemical exposure	L	61	0.722 (0.369)	0.0464 (0.0256)
	M	33	0.746 (0.295)	0.0537 (0.0331)
	H	23	0.702 (0.338)	0.0505 (0.0325)
cancer in family <sup>b</sup>	Y	55	0.731 (0.397)	0.0501 (0.0344)
	N	58	0.716 (0.289)	0.0486 (0.0236)

<sup>a</sup>Excretion values reported as mean (SD)

<sup>b</sup>Responses were not found for this parameter for 4 subjects

<sup>c</sup>Responses were not found for this parameter for 8 subjects

<sup>d</sup>Marginally significant difference among L, M, & H (p<0.10)

NOTE: L = low, M = moderate, H = high

#### 4.2.3 Exposure to Chemicals

Subjects were classified according to their exposure to chemicals (Appendix F) as being low, moderate, or high. For the one-day sample, 52% were classified as low, 28% were classified as moderate, and 20% were classified as high.

The mean and standard deviation for the urinary excretion of conjugated glucuronic acid in mmole per 24 hr and mole in mole creatinine per 24 hr for the one-day sample according to exposure to chemicals is given in Table 11.

#### 4.2.4 Genetics

Subjects were classified according to whether or not any incidence of cancer among their relatives was known. For the one day sample, 49% were classified as having a genetic predisposition to cancer and 51% were classified as not having a genetic predisposition to cancer.

The mean and standard deviation for the urinary excretion of conjugated glucuronic acid in mmole per 24 hr and mole per mole creatinine per 24 hr for the one-day sample according to cancer incidence among relatives is given in Table 11.

#### 4.2.5 Comments

Table 12 shows the between-subject variation in urinary conjugated glucuronic acid excretion in mmole per 24 hr and mole per mole creatinine per 24 hr for the three-day sample comparing all 40 subjects' three-day average excretion values.

Table 13 lists the mean urinary excretion of conjugated glucuronic acid according to certain factors for the 40 subjects. Conjugated glucuronic acid excretion for the factors vegetable intake, fruit intake, and caffeine use were found to be significantly ( $p < 0.05$ ) different among the low, moderate, and high groups when expressed as mole per mole creatinine per day. Additionally, the excretion was significantly ( $p < 0.05$ ) different among low, moderate, and high caffeine users when expressed as mmole conjugated glucuronic acid per 24 hr.

Table 12: Between-Subject Variation in Urinary Excretion of Conjugated Glucuronic Acid For The Three-Day Sample Using Three-Day Average.

<u>Conjugated Glucuronic Acid</u>	Excretion	
	<u>Mean (SD)</u>	<u>CVa</u>
mmole/24 hr	0.848 (0.409)	48.2
mole/mole creatinine	0.0562 (0.0294)	52.4

n = 40

<sup>a</sup>CV = coefficient of variation (%).

Table 13: Urinary Excretion of Conjugated Glucuronic Acid According To Certain Factors For The Three-Day Sample.

Parameter		n	(mmole/24 hr) <sup>a</sup>	(mole/mole Creat/24 hr) <sup>a</sup>
vegetable intake	L	26	0.854 (0.377)	0.0534 (0.0204) <sup>b</sup>
	M	8	0.955 (0.329)	0.0753 (0.0417)
	H	6	0.681 (0.298)	0.0426 (0.0108)
fruit intake	L	31	0.845 (0.359)	0.0525 (0.0192) <sup>b</sup>
	M	6	0.730 (0.218)	0.0547 (0.0267)
	H	3	1.116 (0.552)	0.0972 (0.0606)
meat intake	L	25	0.865 (0.426)	0.0605 (0.0297)
	M	10	0.889 (0.190)	0.0529 (0.0199)
	H	5	0.680 (0.195)	0.0412 (0.0158)
char-broiled intake	L	32	0.846 (0.390)	0.0574 (0.0291)
	H	8	0.857 (0.207)	0.0511 (0.0114)
tobacco use	L	35	0.881 (0.370)	0.0577 (0.0280)
	M	4	0.638 (0.102)	0.0431 (0.0061)
	H	1	0.538 --	0.540 --
alcohol use	L	30	0.858 (0.384)	0.0563 (0.0287)
	M	10	0.818 (0.280)	0.0559 (0.0200)
marijuana use	L	34	0.858 (0.382)	0.0564 (0.0280)
	M	3	0.662 (0.138)	0.0470 (0.0082)
	H	3	0.927 (0.081)	0.0628 (0.0236)
caffeine use	L	20	0.816 (0.285) <sup>b</sup>	0.0616 (0.0307) <sup>b</sup>
	M	7	0.628 (0.182)	0.0406 (0.0128)
	H	9	1.096 (0.532)	0.0613 (0.0249)
chemical exposure	L	19	0.857 (0.458)	0.0507 (0.0217)
	M	11	0.845 (0.125)	0.0601 (0.0219)
	H	10	0.835 (0.344)	0.0621 (0.0382)
cancer in family	Y	20	0.902 (0.437)	0.0609 (0.0325)
	N	20	0.794 (0.257)	0.0514 (0.0185)

<sup>a</sup>Excretion values reported as mean (SD).

<sup>b</sup>Significant difference among L, M, and H (p<0.05).

## CHAPTER V: DISCUSSION

The primary objective of this study was to quantify the urinary conjugated glucuronic acid excretion for a free-living male population. The mean urinary conjugated glucuronic acid excretion for 117 males in this study (Table 7) was approximately 36% of the mean value calculated from Fishman and Green (Table 4): 0.725 vs. 1.99 mmole/24 hr.

It was approximately 35% of the mean value calculated from Mazzuchin et al (Table 5): 0.725 vs. 2.06 mmole/24 hr. The mean urinary conjugated glucuronic acid excretion for the subgroup of 40 males in this study (Table 9) was approximately 43% of that calculated from Fishman and Green (Table 4) and approximately 41% of that calculated from Mazzuchin et al (Table 5).

The upper range values (maximum 1.98 mmole/24 hr) for the 117 males did, however, compare more closely to those calculated from Fishman and Green (maximum 2.38 mmole/24 hr) and also Mazzuchin et al (maximum 2.52 mmole/24 hr).

The published values for glucuronide excretion are also higher than those obtained in this study for the three-day sample of 40 subjects, although this group had a higher mean conjugated glucuronic acid excretion than the one-day

sample of 117 subjects.

Differences between the published values for glucuronide excretion and those obtained in this study could be the result of several factors. Different analytical methods tend to give different measures of glucuronides even from the same sample (69). Mazzuchin et al. (65) point out that there are as many normal values for glucuronide excretion as there are methods.

Most of the values reported in the scientific literature are for small sample sizes. It is possible that for a larger population, the normal range in mg per day of conjugated glucuronic acid excretion is much greater, and that the published data only contained representatives from the upper range.

In this study, most of the 117 subjects excreted less than 200 mg conjugated glucuronic acid per day. However, 15 subjects excreted over 200 mg and of those, 5 excreted 300 mg or more per day.

Another possible reason for the lower values obtained in this study is that of the difficulties with urine collection and storage. Urine contains the enzyme  $\beta$ -glucuronidase, which catalyzes the hydrolysis of the glucuronide conjugate, resulting in the formation of free

glucuronic acid from conjugated glucuronic acid. It is known that bladder  $\beta$ -glucuronidase has a low optimum pH and that acidification of urine causes increased activity of the enzyme (76). Dutton (7) has remarked that the enzyme may be increased in urine during local infection from bacteria and with increased sloughing-off of genito-urinary epithelial cells.

Urines analyzed in this study may have been left standing prior to refrigeration and freezing for critical lengths of time. This would allow cleavage of the conjugated glucuronic acid to occur due to  $\beta$ -glucuronidase activity. Also, contamination during collection may have occurred, resulting in bacterial - glucuronidase being introduced into the urine specimen.

Measurements of bound sulfate excretion from the same subjects (79) were much higher compared to the published data of Lundquist et al. (80). Sulfation and glucuronidation are competing detoxification pathways for many substrates. Caldwell and co-workers (81) have demonstrated a ratio of glucuronidation to sulfation in patients receiving the drug paracetamol (G:S ratio) ranging from 0.35 to 4.64 and a G:S ratio ranging from 0.26 to 4.26 among patients receiving salicylamide. Therefore it

appears possible for sulfation to be the predominant pathway in some instances.

The secondary objective of this study was to examine dietary and other habits of the subjects relative to ingestion or exposure to various food and non-food substances. This information was then compared to the urinary conjugated glucuronic acid excretion. The variables assessed along with brief descriptive information is provided in section 3.4. The statistical F-test was employed to identify any significant differences in conjugated glucuronic acid excretion at different levels of consumption of each variable assessed.

The dietary information obtained from the food frequency questionnaire was useful in a qualitative rather than quantitative sense. From it, the subjects were classified according to frequency of consumption of certain foods as shown in a previous section (4.2.1) as being low, moderate, or high consumers.

For the one-day sample (Table 11) neither vegetable, fruit, meat, nor charbroiled food intake demonstrated significant differences among low, moderate, and high consumers relative to conjugated glucuronic acid excretion measured as mmole per 24 hr or mole per mole creatinine per

24 hr. The three-day sample (Table 13), however, demonstrated a significant difference ( $p < 0.05$ ) in conjugated glucuronic acid excretion (mmole/24 hr and mole/mole creatinine/24 hr) for the vegetable and fruit categories. It should be noted, though, that the sample sizes for the low, moderate, and high categories are greatly diminished in size compared to those for the one-day sample. Based upon the known influence that diet can have on xenobiotic metabolism, the results obtained in this study were not without precedent. Gibson and Skett (76) have summarized the effects of dietary nutrients on drug metabolism (Figure 19).

Of the non-food factors examined, the only category that demonstrated a marginally significant difference in conjugated glucuronic acid excretion among its subgroupings for the one-day data was caffeine use ( $p < 0.1$ ). Figures 20 and 21 present the side-by-side boxplots which schematically represent the variability in conjugated glucuronic acid excretion among caffeine users in mmole per 24 hr and mole per mole creatinine per 24 hr, respectively. For the three-day data, the difference was also significant ( $p < 0.05$ ) among caffeine users. Although each of the remaining non-food factors examined did not explain the

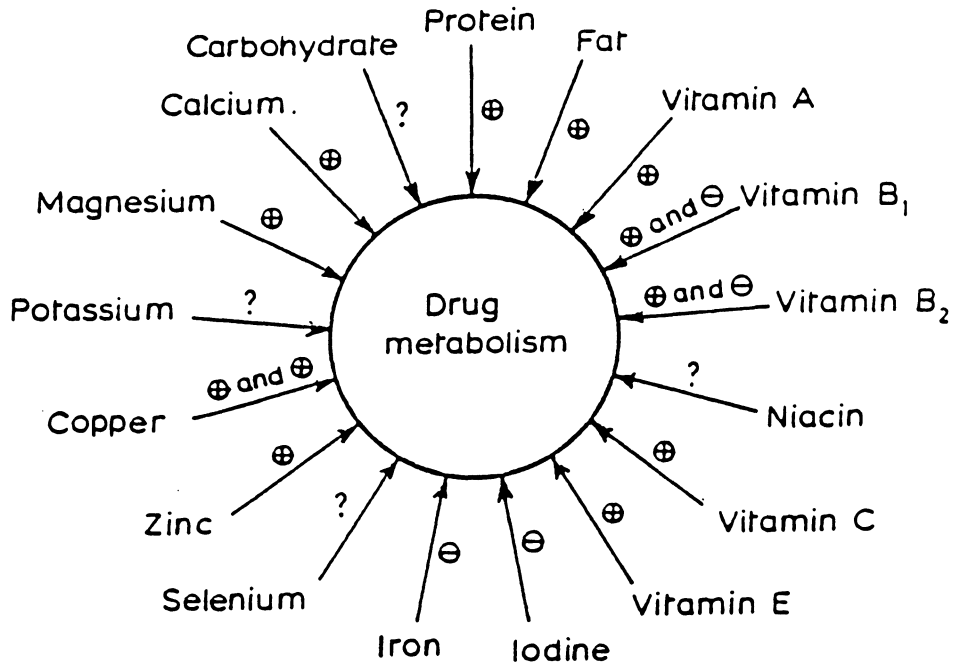


Figure 19: Summary of the effects of dietary nutrients on drug metabolism (76).

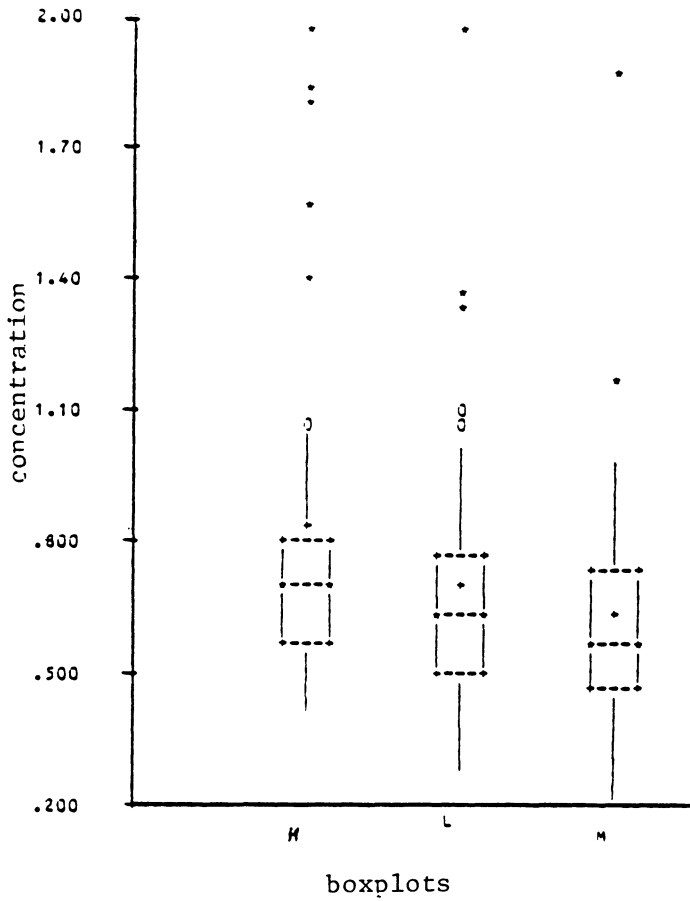


Figure 20: Side-by-side boxplots representative of the variability in urinary conjugated glucuronic acid excretion (mmole/24 hr) among caffeine users (one-day sample; H=high caffeine use; L=low; M=moderate).

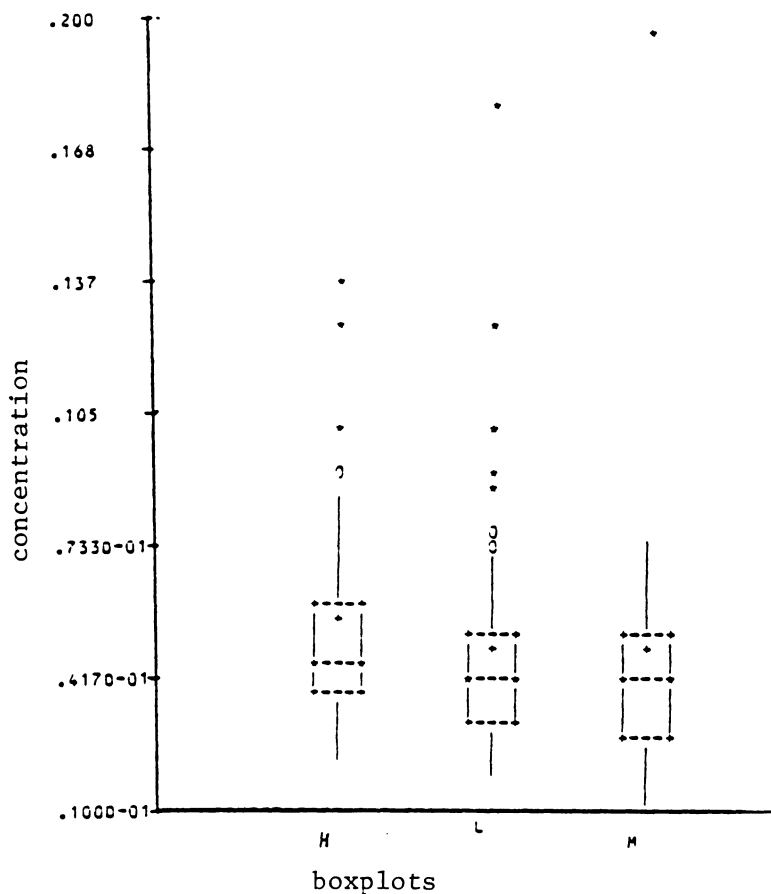


Figure 21: Side-by-side boxplots representative of the variability in urinary conjugated glucuronic acid excretion (mole/mole creatinine) among caffeine users (one-day sample; H=high caffeine use; L=low; M=moderate).

variation in the conjugated glucuronic acid excretion for the one-day and three-day samples, other research has demonstrated the influence of dietary non-nutrients and genetic and environmental factors on xenobiotic metabolism (Figure 22).

The relative importance of physiological and environmental factors in determining the xenobiotic (i.e.: drug) metabolizing capacity in a population is open to debate. Two opposing views exist. From twin and family studies comes the notion that most, if not all, of the differences in drug metabolism in a population are due to genetic differences. Other research has indicated that interindividual variations in drug metabolism can be totally accounted for by environmental factors (including alcohol, caffeine, and tobacco use). The influence of environmental factors in addition to genetic differences is very likely the cause of the interindividual variation seen in a population.

In addition to separate genetic and environmental influences on xenobiotic metabolism, Vesell (82) has suggested the possibility of dynamic interactions among suspected or established dietary factors that may influence drug response in humans. He also has stated that

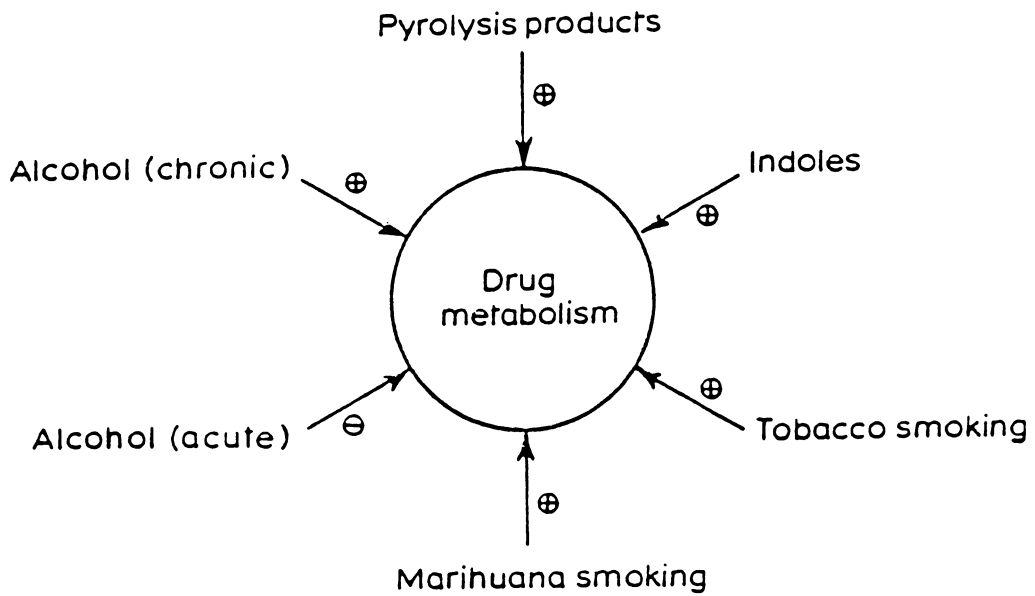


Figure 22: Summary of the influence of dietary non-nutrients on drug metabolism (76).

conflicting results obtained from drug clearance studies resulting in large interindividual variations are not necessarily contradictory due to the multiple genetic and environmental differences between the subjects examined.

In this study, the large interindividual variation most likely masks any effects of diet, environment, or genetics upon urinary conjugated glucuronic acid excretion. The three-day subject means were tested for significance in order to minimize this effect, and differences were noticed in three categories (vegetable intake, fruit intake, and caffeine use). The importance of these findings is unclear due to the small sample sizes in the subgroupings. Even so, it is recognized by this writer that "P" is a mathematical concept that stands for probability and not significance; experiments do not automatically have biological meaning because statistical analysis yields a  $p < 0.05$ . The reason for the large interindividual variation in conjugated glucuronic acid excretion seen in this study is not obvious. Therefore further research is indicated in order to clarify the urinary conjugated glucuronic acid excretion of a free-living population.

I acknowledge the complexity of the subject matter represented by this study, with its many interactive

factors. Hopefully the reader has been stimulated into further examination of one or more aspects of the glucuronidation of xenobiotics.

## CHAPTER VI: SUMMARY AND CONCLUSIONS

The amount of urinary conjugated glucuronic acid excreted by a free-living male population and the effect of certain factors, i.e. vegetable, fruit, meat, and charbroiled food intake, tobacco, alcohol, caffeine, and marijuana use, exposure to chemicals and familial cancer incidence, were investigated. One day of urine from 117 subjects who complied with the collection protocol was analyzed for each subject and three days of urine were analyzed for a randomly selected sub-group of forty subjects.

For the one-day sample, the mean conjugated glucuronic acid excreted was 0.725 mmole/24 hr or 0.0492 mole/mole creatinine. The values for the three-day sample were 0.848 mmole/24 hr or 0.0562 mole/mole creatinine.

An analysis of the one-day data revealed a large degree of between-subject (inter-) variability: 47.1%. The corresponding coefficient of variation for the three-day data was 48.2% when three day averages were compared. The within-subject (intra-) variability for the three-day data corresponded to a coefficient of variation of 29.2%.

The large intervariability probably masked any effects of diet, environment, or genetics upon the observed urinary

conjugated glucuronic acid excretion. Caffeine use, vegetable, and fruit intake did show differences between low, moderate, and high consumers, although the biological importance of these associations for the small sample sizes is questionable. Further research regarding urinary conjugated glucuronic acid excretion and dietary, environmental, and genetic influences is therefore warranted.

## REFERENCES

1. Mason, H.S., North, J.C., and Vanneste, M. Microsomal and mixed function oxidases: the metabolism of xenobiotics. *Fed. Am. Soc. Exp. Biol.* 1965. 24:1172.
2. Neal, R.A. Metabolism of toxic substances. In: Doull, J., Klaasen, C.D., and Amdur, M.O., eds. Casarett and Doull's toxicology, Macmillan Publ. Co. Inc., New York. 1980:56.
3. Williams, R.T. In: Detoxification Mechanisms, Chapman and Hall, London. 1959.
4. Mulder, G.J., Hinson, J.A., and Gillette, J.R. Conversion of the N-O-glucuronide and the N-O-sulfate conjugates of N-hydroxyphenacetin to reactive intermediates. *Biochem. Pharmacol.* 1978. 27:1641.
5. Smith, R.L., and Williams, R.T. In: Dutton, G.J., ed. Glucuronic acid, free and combined, Academic Press, New York. 1966:89.
6. Testa, B., and Jenner, P. In: Drug metabolism: chemical and biochemical aspects, Marcel Dekker, New York. 1976:189.
7. Dutton, G.J. In: Glucuronidation of drugs and other compounds, CRC Press, Boca Raton, Fla. 1980:7.
8. Bidlack, W.R. Toxicant metabolism and the role of nutrients. *Food Technol.* 1982. 10:106.
9. Caldwell, J. Conjugation reactions in foreign-compound metabolism: definitions, consequences, and species variations. *Drug Metab. Rev.* 1982. 13:745-777.
10. Powell, G.M., and Curtis, C.G. Sites of sulphation and the fates of sulphate esters. In: Aitio, A., ed. Conjugation reactions in drug biotransformation, Elsevier/North Holland, Amsterdam. 1978:409.

11. Stoigniew, M., and Fenselau, C. Electrophilic reactions of acyl-linked glucuronides. *Drug Metab. Dispos.* 1982. 10:609.
12. Faed, E. Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab. Rev.* 1984. 14:1213-1249.
13. Bray, H.G., Humphris, B.G., Thorpe, W.V., and Wood, P.B. Kinetic studies of the metabolism of foreign organic compounds. *Biochem. J.* 1952. 52:419.
14. Mulder, G.J., and Scholtens, E. Phenol sulphotransferase and uridine diphosphate glucuronyltransferase from rat liver in vivo and in vitro. *Biochem. J.* 1977. 165:553.
15. Pang, K.S., Rowland, M., and Tozer, N. In vivo evaluation of Michaelis-Menten constants of hepatic drug-eliminating systems. *Drug Metab. Dispos.* 1978. 6:197.
16. Mandel, H.G. In: La Due, B.N., Mandel, H.G., and Way, E.L., eds. Fundamentals of drug metabolism and drug disposition. Williams and Wilkins, Baltimore. 1971:149.
17. Graham, A.M., Woodcock, B.G., and Wood, G.C. The phospholipid dependence of uridine diphosphate glucuronyltransferase. *Biochem. J.* 1974. 137:567-574.
18. Dutton, G.J., and Storey, I.D.E. Uridine compounds in glucuronic acid metabolism. *Biochem. J.* 1954. 57:275-283.
19. Strominger, J.L., Maxwell, E.S., Axelrod, J., and Kalckar, H.M. Enzymatic formation of uridine diphosphoglucuronic acid. *J. Biol. Chem.* 1957. 224:79.
20. Pang, K.S., Koster, H., Halsema, I.C.M., Scholterns, E., and Mulder, G.J. Abberent pharmacokinetics of harmol in the perfused rat liver preparation: sulfate and glucuronide conjugations.

- J. Pharm. Exp. Ther. 1981. 219:134.
21. Axelrod, J., Inscoc, J., and Tomkins, G.M. Enzymatic synthesis of N-glucosyluronic acid conjugates. J. Biol. Chem. 1958. 232:835-841.
  22. Zakim, D., and Vessey, D.A. Membrane dependence of uridine diphosphate glucuronyltransferase. Biochem. Soc. Trans. 1974. 2:1165.
  23. Tomasic, J. The analysis of glucuronic acid conjugates. In: Garret, E.R., and Hirtz, J.L., eds. Drug fate and metabolism, methods and techniques, Marcel Dekker, New York. 1978:283-284.
  24. Levine, W.G. Biliary excretion of drugs and other xenobiotics. Ann. Rev. Pharmacol. Toxicol. 1978. 18:81-96.
  25. 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.
  26. Aitio, A., and Marniemi, J. Extrahepatic glucuronide conjugation. In: Gram, T.E., ed. Extrahepatic metabolism of drugs and other foreign compounds. Spectrum Publ., New York. 1980:365.
  27. Vessey, D.A., and Zakim, D. Regulation of microsomal enzymes by phospholipids. IV. Species differences in the properties of microsomal UDP-glucuronyltransferase. Biochim. Biophys. Acta. 1972. 268:61-69.
  28. Vessey, D.A., and Zakim, D. Regulation of microsomal enzymes by phospholipids. II. Activation of hepatic uridine diphosphate-glucuronyltransferase. J. Biol. Chem. 1971. 246:4649-4656.
  29. Berry, C.S. Critical evaluation of UDP-N-acetylglucosamines as allosteric effectors of UDP-glucuronyltransferase. In: Aitio, A., ed. Conjugation reactions in drug biotransformation. Elsevier/North Holland, Amsterdam. 1978:233-246.

30. Hallinan, T. Comparison of compartmental and conformational phospholipid-restraint models for the intermembranous arrangement of UDP-glucuronyl-transferase. In: Aitio, A., ed. Conjugation reactions in drug biotransformation. Elsevier/North Holland, Amsterdam. 1978:257-267.
31. Conney, A.H. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* 1967. 19:317-366.
32. Notten, W.R.F., and Henderson, P.Th. Alterations in the glucuronic acid pathway caused by various drugs. *Int. J. Biochem.* 1975. 6:111-119.
33. Notten, W.R.F., and Henderson, P.Th. The interaction of chemical compounds with the functional state of the liver. *Int. Arch. Occup. Environ. Hlth.* 1977. 38:197-207.
34. Parke, D. V. The endoplasmic reticulum: its role in physiological functions and pathological situations. In: Jenner, P., and Testa, B., eds. Concepts in drug metabolism, Part B. Marcel Dekker, New York. 1981:1-51.
35. Mason, H.S. Mechanisms of oxygen metabolism. *Science.* 1957. 125:1185-1187.
36. Berry, C., and Hallinan, T. Coupled transglucuronidation: a new tool for studying the latency of UDP-glucuronyl transferase: *FEBS. Lett.* 1974. 42:73-76.
37. Vainio, H. Linkage of microsomal drug oxidation and glucuronidation. In: Karki, N.T., ed. Mechanisms of Toxicity and Metabolism. Pergamon Press, Oxford. 1976:54.
38. Vainio, H. Enhancement of hepatic microsomal drug oxidation and glucuronidation in rat by 1,1,1-tri-chloro-2,2-bis(p-chlorophenyl)ethane (DDT). *Chem.-Biol. Interactions.* 1974. 9:7-14.
39. Vainio, H. Drug hydroxylation and glucuronidation in liver microsomes of phenobarbital-treated rats.

- Xenobiotica. 1973. 3:715-725.
40. Erich, M., Huckle, W., and Larsen, C. Increase in glucuronide conjugation of aflatoxin P<sub>1</sub> after pretreatment with microsomal enzyme inducers. Toxicology. 1984. 32:145-152.
  41. Kamden, L., Magdalou, J., and Siest, G. Effects of aflatoxin B<sub>1</sub> on the action of drug metabolizing enzymes in rat liver. Toxicol. Appl. Pharmacol. 1981. 60:570.
  42. Owens, I.S. Genetic regulation of UDP-glucuronyltransferase: induction by polycyclic aromatic compounds in mice. J. Biol. Chem. 1977. 252:2827-2833.
  43. Vessey, D.A., and Zakim, D., eds. Biochemical Pharmacology and Toxicology Vol. I. John Wiley & Sons, New York. 1985:181.
  44. Alvares, A.P., Anderson, K.E., Conney, A.H., and Kappas, A. Interactions between nutritional factors and drug biotransformations in man. PNAS. 1976. 73:2501-2504.
  45. Kappas, A., Anderson, K.E., Conney, A.H., and Alvares, A.P. Influence of dietary protein and carbohydrates on antipyrine and theophylline metabolism in man. Clin. Pharmacol. Ther. 1976. 20:643-653.
  46. Anderson, K.E., Conney, A.H., and Kappas, A. Nutrition and oxidative drug metabolism in man: relative influence of dietary lipids, carbohydrates, and protein. Clin. Pharmacol. Ther. 1979. 26:4983-501.
  47. Woodcock, B.G. and Wood, G.C. Effect of a protein-free diet on UDP-glucuronyltransferase and sulfotransferase activities in rat liver. Biochem. Pharmacol. 1971. 20:2703-2713.
  48. Mucklow, J.C., Caraher, M.T., Idle, J.R., Rawlins, M.D., Sloan, T., Smith, R.L., and Wood, P. The influence of changes in dietary fat on the clearance of antipyrine and 4-hydroxylation of debrisoquine. British J. Pharmacol. 1980. 9:283.

49. Coon, M.J. Oxygen activation in the metabolism of lipids, drugs, and carcinogens. *Nut. Rev.* 1978. 36:319-328.
50. Yang, C.S. The organization and interaction of monooxygenase enzymes in the microsomal membranes. *Life Sci.* 1977. 21:1047-1057.
51. Hietanen, E., Laitinen, M., Vainio, H., and Hanninen, O. Dietary fats and properties of endoplasmic reticulum: II. Dietary lipid induced changes in activities of drug metabolizing enzymes in liver and duodenum of rat. *Lipids.* 1975. 10:467.
52. Bidlack, W.R., Brown, R.C., and Mohan, C. Nutritional parameters that alter hepatic drug metabolism, conjugation, and toxicity. *Fed. Proc.* 1986. 45:145.
53. Wattenberg, L.W. Studies on polycyclic hydrocarbon hydroxylase of the intestine possibly related to cancer. *Cancer.* 1971. 28:99-102.
54. Loub, W.D., and Loub, W.D. Inhibition of polycyclic aromatic hydrocarbon-induced neoplasia by naturally occurring indoles. *Cancer Res.* 1978. 38:1410-1413.
55. Parke, D.V., and Rahman, H. The induction of hepatic microsomal enzymes by safrole. *Biochem. J.* 1970. 119:53.
56. Pantuck, E.J., Pantuck, E.B., Garland, W.A., Min, B.H., Wattenberg, L.W., Anderson, K.E., Kappas, A., and Conney, A.H. Stimulatory effect of Brussels sprouts and cabbage on human drug metabolism. *Clin. Pharmacol. Ther.* 1979. 25:88-95.
57. Conney, A.H., Pantuck, E.J., Hsiao, K.C., Garland, W.A., Anderson, K.E., Alvares, A.P., and Kappas, A. Enhanced phenacetin metabolism in human subjects fed charcoal-broiled beef. *Clin. Pharmacol. Ther.* 1976. 20:633.
58. Kappas, A.P., Alvares, K.E., Anderson, E.J., Pantuck, R., Chang, R., and Conney, A.H. Effect of charcoal-broiled beef on antipyrine and theophylline

- metabolism. Clin. Pharmacol. Ther. 1978. 23:445-450.
59. Minnigh, M.B., and Zemaitis, M.A. Altered acetaminophen disposition in fed and food-deprived rats after acute ethanol administration. Drug Metab. Dispos. 1982. 10:183-188.
  60. Reidenberg, M.M., and Vesell, E.S. Unaltered metabolism of antipyrine and tolbutamine in fasting man. Clin. Pharmacol. Exp. Ther. 1975. 17:650-656.
  61. Mehta, S., Kalsi, H.K., Jayaraman, S., and Mathur, S. Chloramphenicol metabolism in children with protein caloric malnutrition. Am. J. Clin. Nutr. 1975. 28:977-981.
  62. Fishman, W.H., and Green, S. Microanalysis of glucuronide glucuronic acid as applied to  $\beta$ -glucuronidase and glucuronic acid studies. J. Biol. Chem. 1955. 215:527-537.
  63. Fishman, W.H., and Yuki, H. A carbazole method for the differential analysis of glucuronate, glucosiduronate, and hyaluronate. Biochim. Biophys. Acta. 1963. 69:576-578.
  64. Dische, Z. A new specific color reaction of hexuronic acids. J. Biol. Chem. 1947. 167:189-198.
  65. Mazzuchin, A., Walton, R.J., and Thibert, R.J. Determination of total and conjugated glucuronic acid in serum and urine employing a modified naphthoresorcinol reagent. Biochem. Med. 1971. 5:135-157.
  66. Kamil, I.A., Smith, J.N., and Williams, R.T. Studies in detoxication. SU. The isolation of methyl and ethyl glucuronides from the urine of rabbits receiving methanol and ethanol. Biochem. J. 1951. 50:235.
  67. Schachter, D. The chemical estimation of acyl glucuronides and its application to studies on the metabolism of benzoate and salicylate in man. J. Clin. Invest. 1957. 36:297-302.

68. Wagner, G., and Hollmann, S. New enzymatic method for determination of free and conjugated glucuronic acid. *J. Clin. Chem. Clin. Biochem.* 1976. 14:225-226.
69. Klopff, L.L., and Nieman, T.A. Determination of conjugated glucuronic acid by combining enzymatic hydrolysis with lucigenin chemiluminescence. *Anal. Chem.* 1985. 57:46-51.
70. DiCarlo, F.J. Metabolism, pharmacokinetics, and toxicokinetics defined. *Drug Metab. Rev.* 1982. 13:1-4.
71. Vesell, E. Effect of dietary factors on drug disposition in normal human subjects. In: Finley, J.W., and Schwass, D.E., eds. Xenobiotic metabolism: Nutritional Effects, Amer. Chem. Soc., Wash., D.C. 1985:61-75.
72. Anon. Tools for nutrition counseling. In: Bennion, M., ed. Clinical nutrition. Harper and Row, New York. 1979:29-52.
73. Beal, V.A. The nutritional history in longitudinal research. *J. Am. Diet. Assoc.* 1967. 51:426-432.
74. Anon. Dietary methodologies. In: Christakis, G., ed. Nutritional assessment in health programs. *Am. J. Publ. Health Suppl.* 1973. 63:11-15.
75. 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.
76. Gibson, G.G., and Skett, P. Introduction to Drug Metabolism. Chapman and Hall, London. 1986. p.142.
77. 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.
78. Graham, D.M. Caffeine-its identity, dietary source, intake, and biological effects. *Inter. Rev.* 1978. 36:97-102.

79. Robichaud, V. The urinary excretion of sulfoconjugates in an adult male population. Masters Thesis, Department of Human Nutrition and Foods, V.P.I. & S.U., Blacksburg, Virginia. June, 1986.
80. Lundquist, P., Martensson, J., Sorbo, B., and Ohman, S. Turbidimetry of inorganic sulfate, ester sulfate, and total sulphur in the urine. Clin. Chem. 1980. 26:1178-1181.
81. Caldwell, J., Davies, S., Boote, D.J., and O'Gorman, J. Interindividual variation in the sulfation and glucuronidation of paracetamol and salicylamide in human volunteers. In: Sulfate Metabolism and Sulfate Conjugation, edited by Mulder, Caldwell, VanKenpen, and Bonk; Taylor and Francis LTD, London. 1982.
82. Vesell, E.S. Effect of dietary factors on drug disposition in normal human subjects. In: Xenobiotic Metabolism: Nutritional Effects. Finley, J.W. and Schwass, D.E., eds. Amer. Chem. Soc. Washington, D.C. 1985. p. 61-75.

## APPENDICES

APPENDIX A  
INFORMED CONSENT FORM FOR  
PARTICIPATION IN NUTRITION RESEARCH CONDUCTED  
BY THE HUMAN NUTRITION AND FOODS DEPARTMENT  
AT VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

---

You are invited to participate in the Human Detoxification Profile Study. Our goal is to determine the relative ability of adult males to detoxify non-specific, non-nutritive substances, commonly referred to as Xenobiotics. Please read the following information which outlines the specifications of this research experiment.

I. SUBJECT REQUIREMENTS:

You must be a healthy adult male who is not currently taking any prescribed medication and who is not currently under a physician's care.

II. PROCEDURES:

- A. FOOD FREQUENCY QUESTIONNAIRE: You will be required to complete a food frequency questionnaire, identical to the one submitted at the Pre-Experimental screening session.
- B. URINARY SAMPLES: You will be required to collect all urinary excretion produced during a consecutive three day period. The collection will consist of three, 24-hour samples, to be submitted to the lab at the end of each 24-hour period. You will be provided with the necessary urine bottles and complete instructions for collection and proper handling.

III. POSSIBLE RISKS:

The procedures involved in this study are approved by the Institution Review Board for Research involving Human Subjects, and are considered to involve "minimal risk."

IV. POSSIBLE BENEFITS:

Since this study is designed for research purposes, possible benefits from your individual participation include the personal satisfaction of contributing to the body of scientific information which may ultimately prove beneficial to society and determining the relative ability of individuals to detoxify xenobiotics.

V. COMPENSATION:

For your participation and complete cooperation, you will be offered a cash payment of \$10.00, payable upon completion of your involvement in the study.

VI. YOUR RIGHTS:

- A. You have the right to confidentiality. All information obtained during this study that can be identified with you, shall remain confidential.
- B. You have the right to expect an honest answer to any questions that you may have at anytime during the study.
- C. You are free to withdraw from the study at any time, without prejudice.
- D. You have the right to receive a copy of this document.

Your signature indicates that you have agreed to participate in accordance with the conditions described in the preceeding pages.

I have read, and fully understand this consent document. All of my questions have been answered to my satisfaction and I agree to participate in the study.

---

 DATE

---

 SIGNATURE OF PARTICIPANT

---

 SIGNATURE OF WITNESS

---

 SIGNATURE OF INVESTIGATOR(S)

Please contact the following people if you have any additional questions:

1. Dr. Ryland E. Webb, Principal Investigator  
Department of Human Nutrition and Foods  
Phone Number
2. Dr. Forrest W. Thye  
Department of Human Nutrition and Foods  
Phone Number
3. Graduate Students: Jane Santi and Vernice Robichaud  
Phone Number

APPENDIX B  
DETOXIFICATION PROFILE STUDY 1984

Xenobiotics are compounds which do not have nutritive value. Common examples of xenobiotics are caffeine, tobacco, and a host of compounds found in common foods. These compounds undergo a series of reactions in the body in order for them to be rendered less toxic and to be excreted. Most research to date has investigated the various detoxication pathways by observing how a specific compound is metabolized and how long it takes for excretion to occur. This study will measure the end products of detoxication, i.e. conjugates, that are excreted in the urine in order to establish a profile of your detoxification capability.

Subjects will be required to complete a food frequency survey (no quantities or measurements are involved) and to collect their urine for 3 days. You must be on a weight maintenance program, i.e. any weight loss or gain during the collection period would jeopardize the validity of your data. Regular food habits and levels of activity are strongly encouraged during this period. Remember, this study is designed to assess your detoxification capability under normal conditions.

If you have any questions, please do not hesitate to contact Jane or Vernice at 961-5840 between the hours of 9:00 am and 12:00 NOON.

Thank you for your time and consideration.

## APPENDIX C

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 _____	g. Maternal grandparent(s) _____
b. Father _____	h. Paternal grandparent(s) _____
c. Brother(s) _____	i. Aunt(s) _____
d. Sister(s) _____	j. Uncle(s) _____
e. Son(s) _____	k. First cousins _____
f. Daughter(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	e. 1-2 cup(s)/day
b. 1-2 cup(s)/week	f. 3-5 cups/day
c. 3-4 cups/week	g. 6-10 cups/day
d. 5-6 cups/week	h. more than 10 cups/day
3. If coffee was consumed, describe the kind most frequently used.
 

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



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

<u>Cigarettes</u>	<u>Cigar</u>	<u>Pipefills</u>
a. less than 1/day	a. less than 1/day	a. less than 1/day
b. 1-5/day	b. 1/day	b. 1/day
c. 6-10/day	c. 2/day	c. 2/day
d. 11-15/day	d. 3/day	d. 3/day
e. 16-20/day	e. 4/day	e. 4/day
f. 21-25/day	f. 5+/day	f. 5+/day
g. 26-30/day		
h. 30+/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.)

	<u>Monthly</u>	<u>Weekly</u>	<u>Daily</u>
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.

	USED, But 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. Inhalants (glue)						
4. Heroin						
5. Marijuana or Hash						
6. Stimulants (Amphetamines, diet pills, speed)						
7. Tranquilizers (Valium, quaaludes, phenobarbital)						
8. Other Narcotics (Codeine, Opium)						



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 PRE- VENT EXPOSURE (VERY HIGH)	DAILY OR ALMOST DAILY EXPOSURE. GREAT PRECAU- TION TAKEN TO PREVENT EXPO- SURE (HIGH)	WEEKLY EXPOSURE OR LESS. LOW PRECAUTION TAKEN TO PRE- VENT EXPOSURE (MEDIUM)	WEEKLY EXPOSURE OR LESS. GREAT PRECAUTION TAKEN TO PRE- VENT EXPOSURE (LOW)
a				
b				
c				
d				
e				
f				
g				
h				
i				
j				





## APPENDIX D

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

-3-

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
Popecorn	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
Matzo or Ovster	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



## APPENDIX E

## CREATININE

## GENERAL DESCRIPTION

The most frequently used analytical procedure for Creatinine is based on the reaction between alkaline picrate and creatinine (Jaffe reaction).

The automation of the creatinine technique was accomplished by:

1. D.L. Stevens and L.T. Stoggs
2. A.L. Chasson, H.T. Grady and M.A. Stanley

The sample stream, segmented with air, is diluted with 1.8% Sodium Chloride. This combined stream is mixed after which it enters the donor side of the dialyzer. The analytical stream consists of water segmented with air. After emerging from the dialyzer, it is joined with 0.5 N NaOH. These two components are mixed and then joined with Picric Acid. The three components are mixed, and phased to the Colorimeter. Absorbance of the analytical stream is measured at 505 nm ( $m\mu$ ) in a 15 mm flowcell.

TIMEPAC REAGENTS<sup>™</sup>

Reagents necessary for operation of the Auto-Analyzer II Creatinine procedure for a minimum of 12 hours operating time, are supplied by Technicon in one convenient to use package containing appropriate proportions for the operating time indicated.

TIMEPAC CREATININE REAGENT, Technicon No. T40-0004.

## PREPARED REAGENTS

Technicon also supplies the following packaged reagents for use in this method:

SODIUM CHLORIDE 1.8% Technicon No. T01-0385.

SODIUM HYDROXIDE, 0.5N Technicon No. T01-0044.

PICRIC ACID (Saturated) Technicon No. T-01-0043.

BRIJ-35, 30% Solution, Technicon No. T21-0110

## REAGENTS

SODIUM CHLORIDE 1.8% (Technicon No. T01-0385)

Chemical Composition:

Sodium Chloride	18.0 g
Distilled water, q.s.	1000.0 ml
Brij-35, 30% Solution (Technicon No. T21-0110)	0.3 ml

Preparation:

1. Place approximately 500 ml of distilled water in a 1-liter volumetric flask.
2. Add 18.0 g of Sodium Chloride, and shake the flask until the Sodium Chloride is completely dissolved.
3. Dilute to volume with distilled water, and mix.
4. Add 1.0 ml of Brij-35, 30% Solution and mix.

SODIUM HYDROXIDE, 0.5 N

(Technicon No. T01-0044)

Chemical Composition:

Sodium Hydroxide	20.0 g
Distilled water, q.s.	1000.0 ml

Preparation:

1. Place approximately 800 ml of distilled water in a 1-liter volumetric flask.
2. Add 20.0 g of Sodium Hydroxide, and shake the flask until the Sodium Hydroxide is completely dissolved.
3. Dilute to volume with distilled water, and mix.

PICRIC ACID (Saturated) (Technicon No. T01-0043)

Chemical Composition:

Picric Acid (reagent grade)	13.0 g
Distilled water, q.s.	1000.0 ml

Preparation:

1. Place 13.0 g of Picric Acid in a 1-liter volumetric flask.
2. Dilute to volume with distilled water.

3. Allow the excess Picric acid to remain in contact with the water and shake occasionally.
4. Filter, and store in a polyethylene bottle.

**CREATININE WATER**

**Chemical Composition:**

Distilled water	1000.0 ml
Brij-35, 30% Solution (Technicon No. T21-0110)	0.3 ml

**Preparation:**

1. Place 1000 ml of distilled water in a 1-liter container.
2. Add 1.0 ml of Brij-35, 30% Solution, and mix.

**GENERAL NOTES**

1. The Creatinine procedure can be run at 60 determinations per hour using single point standardization with the Digital Printer.
2. The range for this method is 1-20 mg%. Sera with values over 20 mg% can be diluted with distilled water and re-run.

3. Samples should consist of clear, unhemolyzed serum.

4. All sera should be well centrifuged and carefully decanted to avoid introducing clots into the system.

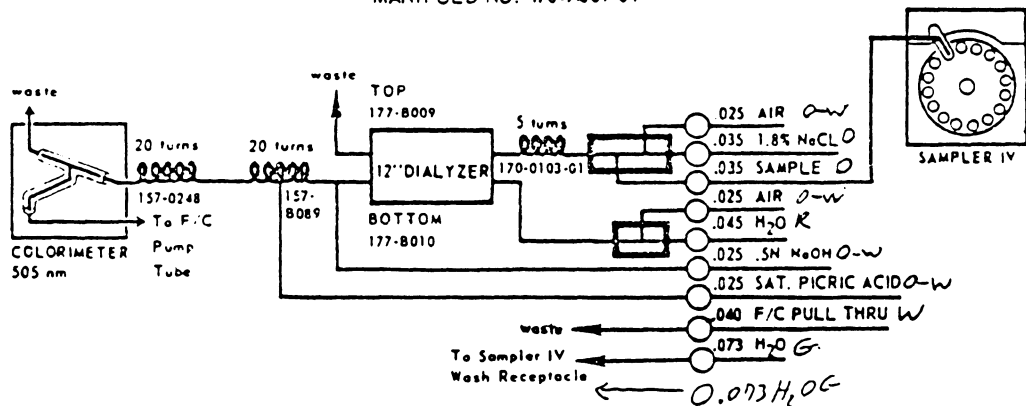
5. If noisy records are obtained, it may be due to the formation of precipitate. If such is the case, cleaning the picric acid and NaOH lines as well as the coils with 10% acetic acid will probably resolve the problem.

6. At the end of each working day, the system should be flushed with distilled water for 15 minutes. If the Technicon Timepac is being used, this can be accomplished by placing the valve in the wash position.

7. Before running this method, switch the range on the Digital Printer to 200; set the mode switch in the Normal position; set the Sampling Rate switch to 60, and place the Decimal Switch in the 00.0 position. (See Instruction Manual TA0-0170-00).

8. If the Technicon Timepac is not being used with the system, it may be advantageous to add filters to the reagent lines.

**CREATININE AA 11-11**  
MANIFOLD NO. 170-A009-01



## APPENDIX F - Exposure of subjects to chemical substances

F. Environmental NA=70

<u>Code #</u>	<u>Substance</u>	<u>Exposure</u>	<u>Exposure in last two weeks *</u>
1	Blue-Print Ammonia Vapor	Very High	*
	D-76 Photo Developer	Medium	*
	Photo Fixer Soln.	Medium	*
	Dektol Photo Developer	Medium	*
	Stop Bath - Photo Soln.	Medium	*
	Gasoline	Medium	*
2	Ajax Detergent	Very High	*
	Dishwasher Detergent	Very High	*
3	Gasoline	Medium	*
4	Roach Insecticide	Low	
5	Gasoline	Low	*
8	Swimming Pool Chemicals	Very High	*
11	Windex	Very High	*
12	Lacquer Spray Paint	Medium	
	ETOH	Low	
	Ether	Low	*
13	Gasoline	Very High	*
	Motor Oil	Very High	*
	Spray Paint	Very High	*
14	Gasoline	Medium	*
	Grease solvents	Very High	*
	Acetone	Medium	
	Motor Oil	Very High	*
15	Coal Ash	Very High	
	Fresh. Chem. Lab.	Medium	
16	Paint Thinner	Medium	*
	Varathane Paint	Medium	
19	Spray Paint	Medium	*
22	Motor Oil	Medium	*
	Gasoline	Low	*
23	Incense	Medium	*
24	Epoxy Glue	Medium	
	Incense	Medium	*

<u>Code #</u>	<u>Substance</u>	<u>Exposure</u>	<u>Exposure in last two weeks *</u>
25	ETOH	Medium	*
	Acetic Acid	Medium	*
	NaOH	Medium	*
28	Gasoline	Low	*
	Paint	Medium	*
31	Hexachlorophine Topical	Very High	*
32	Gasoline	Low	*
33	Brasso	Very High	*
	Shoe Polish	Very High	*
	Tenacitin	Medium	*
	Windex	Medium	*
35	Gasoline	Low	
36	Gasoline	Low	
37	Gasoline	Low	*
38	Organic Chem. Lab.	Low	*
39	Chem. Lab.	Low	*
	Carbon Monoxide	Medium	
40	Acetone	Medium	*
	Ether	Medium	*
	Benzene Derivatives	Medium	*
	Pyridine	Medium	*
41	Burnt Coal (Power Plant)	High	*
42	Acetone	Medium	
	Benzene	Medium	
	Xylene	Medium	
44	Gasoline	Very High	*
45	Acetone	Medium	*
	Ether	Medium	*
	Pyridine	Medium	*
	Benzene Derivatives	Medium	*
47	Shoe Polish	Very High	*
	Shoe Edge Dressing	Very High	*
	Brasso	Very High	*

<u>Code #</u>	<u>Substance</u>	<u>Exposure</u>	<u>Exposure in last two weeks *</u>
48	Exhaust Fumes	Medium	*
49	Motor Oil	Very High	*
	Grease	Very High	*
	Exhaust	Very High	*
56	CCl <sub>4</sub>	Low	
61	Polyurethane	Low	*
	Wood Stain	Low	*
	Paint (Model)	Low	*
	Spray Paint	Low	*
	Wood Glue	Low	*
65	Gen. Chem. Lab.	Low	
68	Crazy Glue	Low	*
72	Hydrogen Sulfide	Medium	*
	Ammonia	Medium	*
	Spray Paint (Krylon)	Medium	*
74	Gasoline	Medium	*
78	Gen. Chem. Lab.	Medium	
80	Fireplace Smoke	Very High	
	Lighter Fluid	Medium	
82	Gasoline	Low	*
	Printer's Solvent	Low	
83	Gasoline	Medium	
87	Xerox Printer's Dry Ink	Medium	*
88	Gasoline	Medium	*
89	Gasoline	Medium	*
	Kerosine	Medium	
	Paint	Medium	
90	Gasoline	Medium	*
92	Acetone	Low	
	Ammonia	Very High	*
93	Roach Pesticide	Medium	*

<u>Code #</u>	<u>Substance</u>	<u>Exposure</u>	<u>Exposure in last two weeks *</u>
94	Gasoline	Low	*
96	Gasoline Battery Acid	Medium Medium	*
97	Oil Based Paints	Very High	
104	Gasoline	Low	*
107	Gasoline Paint	Medium Low	*
120	Gasoline Bike Patch Cement Kerosene House Paint	Low Low High Low	* * * *
121	Gasoline	Medium	*
122	Benzene Hexene Chem. Lab.	Low/Medium Low/Medium Low/Medium	* * *
123	Gasoline Latex Paint	Low Medium	* *
124	Gasoline	Medium	*
126	Paint Remover	Medium	*
127	Chem. Lab. (HCl, K <sub>2</sub> CrO <sub>4</sub> , SnCl <sub>2</sub> , H <sub>2</sub> O <sub>2</sub> , HNO <sub>3</sub> , NH <sub>4</sub> OH) Gasoline	Low Medium	* *
136	Paint	Very High	*
138	Ammonia Vapor	Very High	*
139	Gasoline	Medium	*
140	Gasoline Coal Cologne	Medium Low Very High	* * *
141	Wood Stain Gasoline Fumes	Very High Very High	* *
142	Chem. Lab. Technician	High	*

<u>Code #</u>	<u>Substance</u>	<u>Exposure</u>	<u>Exposure in last two weeks *</u>
144	Gasoline Wall Paint Ammonia	Medium Very High High	* * *
146	Liquid Ammonia	Medium	
147	Gasoline	Medium	*
154	Gasoline Motor Oil Chlorohexane CCl <sub>4</sub> Na Thiosulfate Model Glue	Medium Medium Low Low Low Medium	  * * * *
157	Acetone Methanol Ethanol Hexanes Chloroform	High High High High High	* * * * *
158	Triethylamine Grease Solvent	Medium Medium	* *
160	Gasoline	Medium	
161	CCl <sub>4</sub> Gasoline Paint	Medium Low Low	* * *
163	Lime (Concrete Mix)	Medium	
166	Gasoline	Low	*
168	Oil based Paints	Very High	
175	Chem. Lab.	Medium	*
176	Gasoline	Medium	*
177	Gasoline	Medium	
180	Gen. Chem. Lab.	Low	*
183	Gasoline	Medium	
190	CCl <sub>4</sub> Gasoline	Medium Medium	 *

<u>Code #</u>	<u>Substance</u>	<u>Exposure</u>	<u>Exposure in last two weeks *</u>
190	Super glue	Medium	
	Ammonia	Medium	*
192	Suntan Oil	Medium	*
194	Acetone	Very High	*
	CCl <sub>4</sub>	High	*
	Malathion	Medium	
	Diazinon	Medium	*
	PCB	Medium	*
	Phenol	Low	*
	Benzene	Very High	*
	T-Butyl Alcohol	Very High	*
195	HCl	Medium	
	HNO <sub>3</sub>	Medium	
	NH <sub>4</sub> OH	Medium	
199	Varnish	Low	*
	Wood Stain	Low	*
201	Wood Stain	Medium	*
	Polyurethane	Medium	*
	Chem. Lab.	Low	*
204	Acetone	Medium	*
	Methyl Alcohol	Medium	
	Safranin	Medium	*
	Crystal Violet	Medium	
	Gasoline	Low	*
	Motor Oil	Low	*
205	HCl	Low	*
	Nitric Acid	Low	*
	Ammonium Hydroxide	Low	*
	Mercury	Low	
	Acetic Acid	Low	
	Ammonia	Low	-*
	Lead Dioxide	Low	
	Sodium Hydroxide	Low	*
206	HCl	Low	*
	Nitric Acid	Low	*
	Ammonium Hydroxide	Low	*
	Mercury	Low	
	Acetic Acid	Low	
	Ammonia	Low	*
	Lead Dioxide	Low	
	Sodium Hydroxide	Low	*

F.8.

- # 1 Temin, Benuate, Diazinon, Metasystox-R, Pirimor, Dipel, Banrot, Rencap, Pentac, B-9, Round-up, Paraquat, Malathion, Triforene, Sevin, Lannate (Very High)
- # 6 Exterior Spray Paint, Herbicides, Insecticides, Murionic Acid (Very High)
- # 27 Atrazine, Gasoline, Tordon, Etc. (Very High)
- # 35 Oil-Based Paints (High)
- # 43 Oil-Based Paints (Very High)
- # 66 Oil-Based Paints (Very High)
- # 78 Deverane Paint (Very High)
- # 93 "Safety Kleen" Auto Parts Degreaser Solvent (High)
- # 97 Cadmium Yellow (High)
- #127  $Mn_3O_4$ , MoCap, Sucker Pucker, Sevin, Malathion (High)
- #143  $CCl_4$ , Benzyl Alcohol, Benzene, Film Processing Cupds. (Very High)
- #157 Oil-Based Paint (High)
- #168 Oil-Based House Paint (Very High)
- #169 Oil-Based Paints, Pesticides (High)
- #192 Gasoline, Air Freshner (High)
- #194 Diazinon, Malathion, Chloradane, DDT (High)
- #199 Electroplating Acids (High)
- #209 Round-UP Herbicide (High)

<u>Code #</u>	<u>Substance</u>	<u>Exposure</u>	<u>Exposure in last two weeks *</u>
209	Round-Up Herbicide	High	
210	Acetone	Medium	*
	Diethylether	Medium	*
	Carbonyl Cmpds	Medium	*
	Ethanol	Medium	*
212	Acetone	Medium	*
	HCl	Medium	*
	HNO <sub>3</sub>	Medium	*
	NH <sub>4</sub> OH	Medium	*
	Stain	Low	*
	Freon	Low	*
	Gasoline	Low	

The vita has been removed  
from the scanned document