QUANTITATIVE ASSESSMENT OF DAILY URINARY CONJUGATES IN AN ADULT MALE POPULATION

by

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(ABSTRACT)

The effect of a self-selected and semisynthetic diet on urinary conjugates levels was determined in 18 male adults (22-40 y). Urinary conjugates were also quantified to develop an index of detoxification using a multivariate approach. The four major urinary conjugates measured were glucuronides, sulfoconjugates, mercapturates, and amino acid conjugates. Subjects consumed a self-selected diet for three days and a semisynthetic diet for seven days. Mercapturates and amino acid conjugates were most affected by dietary change, excretion levels reduced by about 50% during the semisynthetic diet period (0.27±0.11 vs 0.14±0.02 mmol/24-h; 5.99 vs 3.03 mmol/24-h, respectively). Glucuronides were the least responsive to dietary change with no significant difference between the means of the two diet periods (self-selected diet 2.93±0.77; semisynthetic 3.21±0.29 mmol/24-h). Four methods for developing 'normal' ranges were presented: mean±SD; percentiles; principal component analysis (princomp); Mahalanobis distance (distquan). The four methods were compared. In summary, conjugate excretion levels were found to be sensitive to dietary changes, with some pathways more responsive than others. Also, the princomp and distquan methods were stressed because they are a multivariate approach which combine values for all three pathways and their interaction into a single value that would then be representative of an individual's total, or overall, detoxification level relative to the others in this group.

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May I extend an invitation to all these people to come to Kenya as my visitors. Hope to meet there!

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JOURNAL ARTICLE I

INTRODUCTION

A wide variety of chemicals are required for normal growth, maintenance, and reproduction in mammals. Ingestion, the mode by which mammals acquire these nutrients, carries with it the risk of exposure to other naturally occurring and synthetic chemicals that may be toxic. These potentially toxic chemical compounds have no apparent nutritive value and are collectively named xenobiotics (1-3). How readily these compounds are excreted largely depends on their water solubility (2). Compounds that are more soluble in lipid-like materials than in water tend to accumulate in the mammalian organism and this accumulation may lead to toxicity. Therefore, to facilitate excretion of these compounds after entering the body, they undergo metabolic changes which are for the most part designed to reduce toxicity and facilitate urinary excretion.

The detoxification of compounds foreign to the intermediary metabolism of mammals is a process that involves a series of metabolic conversions catalyzed by enzymes, a number of nonspecific reactions, and final excretion (2,4,5). The commonly accepted view is that the metabolic pathways encountered in detoxification represent a series of steps by which foreign compounds are "prepared" for excretion. Williams (4) suggested that the metabolism of xenobiotics can be thought of as occurring in two major phases. Phase I reactions involve oxidation, reduction, and/or hydrolysis and phase II consist of conjugation reactions with endogenous compounds (3,5,6). The phase I reactions generally convert the xenobiotics to derivatives that can undergo phase II reactions (5,6). Thus, phase I reactions both enhance the water solubility of the xenobiotics and, also, generate a substrate for phase II metabolism (6,7). A detailed discussion of phase I metabolism, by Pritchard and James, can be found in Jakoby, Bend and Caldwell (5) and an equally detailed discussion in Gibson and Skett (6).

Phase II metabolism (or conjugation) involves a diverse group of enzymes

acting on disparate types of compounds. Customarily, 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 (6,8,9). In general, these reactions lead to a water-soluble product which can be excreted in bile or urine and serve to end the biological activity of the compound (6,9). Thus, the formation of conjugates plays an important role in the metabolism of a variety of drugs and other xenobiotic compounds. The type of conjugate formed depends on the reactive group in the compound and availability of conjugating groups.

Of the seven major groups of conjugation reactions listed by Caldwell (8), four are of major concern in humans. These four include glucuronidation, sulfation, amino acid conjugation, and glutathione conjugation. Methylation and acetylation are of lesser significance in xenobiotic detoxication since both types of conjugations do not increase the polarity of the substrate. Glucoside conjugation is of little known importance in mammals. The common feature of all the pathways involves the conjugation of an endogenous molecule to a functional group on the xenobiotic substrate.

Conjugation with glucuronic acid is catalyzed by microsomal UDP-glucuronyl transferase (GT, EC 2.4.1.17) and is a major pathway by which the mammalian organism inactivates and eliminates a wide variety of lipid-soluble xenobiotics and endogenous compounds such as steroids and bilirubin (8,10). Glucuronidation is perhaps the most versatile of the conjugation reactions because of the range of molecular structures glucuronidated. It involves the enzymatic transfer of the glucuronic acid moiety from UDP-glucuronate to the xenobiotic compound with subsequent excretion via the kidneys or bile. Excretion via the bile occurs with amphipathic glucuronides whose molecular weight is approximately 500 or over (9).

In sulfate conjugation, the xenobiotic is sulfated by transfer of the active sulfate of phosphoadenosine phosphosulfate. Although sulfation generally occurs

with phenols, other hydroxyl groups, amino groups, and even phosphates and thiol groups may be sulfated (8). The enzymes involved in sulfation are widely distributed in the tissues and quantitatively sulfate formation is more extensive when compared to glucuronide formation (8).

Glucuronide and sulfate pathways may be regarded as competing pathways. However, of these two routes of conjugation, sulfation is more likely to be limited. Not only does sulfation require higher energy expenditure but also endogenous sulfate is usually more limited than endogenous glucuronic acid. Sulfate conjugation tends to predominate at low xenobiotic levels and with compounds having small, chemically simple structures. With increasing xenobiotic levels or decreasing sulfate levels the ratio of glucuronidation to sulfation increases (11).

Conjugations with amino acids are mainly reactions with aromatic carboxylic acids. The carboxylate group is activated to a CoA thioester followed by transfer of the activated acyl moiety to the amino acid (2,6,8). In the human the major amino acid conjugates involve the amino acids glycine and glutamine (6). The amino acid pathway of conjugation is complementary to glucuronidation in that xenobiotics may be metabolized by either or both pathways (6,8,12).

The nucleophilic tripeptide glutathione (glutamyl-cysteinyl-glycine) conjugates with a variety of xenobiotics, and because of their molecular weight and amphiphilic character, are excreted preferentially via the bile (12). Glutathione conjugates are rarely found in the urine. However, glutathione conjugates do undergo extensive metabolism of the peptide moiety with the ultimate formation of S-substituted N-acetylcysteines or mercapturic acids with subsequent urinary excretion (6,8,13).

It is obvious from the preceding discussion that ingested xenobiotics (drugs, nonnutrients, etc.) can be metabolized by a variety of chemical pathways catalyzed by different enzyme systems. As with other metabolic pathways, these detoxification reactions do not usually occur at random. Specific biological control mechanisms are operative at several stages in the overall process (6). Additionally, these metabolic

pathways do not normally function in isolation - the activity of one pathway can influence the activity in another. Moreover, many drug biotransformation pathways are intimately related to endogenous metabolic pathways, both sharing the same source of co-factors, co-substrates, prosthetic groups and even enzyme systems (6). This leads to competition for available enzyme between the xenobiotic and the endogenous substrate. As many endogenous substrates are in a constant state of flux due to synthesis, utilization and degradation reactions, this competition can influence the availability of the enzyme (e.g., due to saturation) for detoxification reactions (6).

Conjugates are the major excretory products for the vast majority of xenobiotics. The significance of conjugation as a detoxification mechanism becomes obvious only when the reaction is impaired. Saturation or a metabolic defect of the enzyme system can cause the protective effect of the system to be voided and toxicity may be manifested in some manner (7,14). Subsequently, the conjugation reactions are generally regarded as true 'detoxification' reactions and changes in the ability of the body to conjugate xenobiotics leads to large changes in toxicity of the xenobiotic (6,12). Thus, this research will attempt to determine an individual's index of detoxification by measuring conjugate levels in urine.

In view of our current knowledge of the complexities of xenobiotic metabolism, as reflected by phase I and phase II enzymatic reactions, it would appear feasible to assess the detoxification capability of the body by measuring the conjugates, which reflect the various pathways of detoxification, excreted in the urine. Therefore, the overall purpose of this study is to determine the detoxification capability of humans by measuring the urinary endproducts of four different conjugation pathways. To determine an individual's capability to detoxify, experimental procedures are designed to accomplish the following specific objectives:

1) To determine the usual urinary level of conjugates of four different pathways and the sum of these pathways.
2) To determine the endogenous level, i.e., in the absence of dietary

nonnutrients, of the four pathways and their sum by feeding a semisynthetic diet.

3) To determine the time required to attain endogenous levels of the four pathways and their sum.

4) To determine the influence of diet on the variation in endogenous levels of the four pathways.

5) To index individuals into high and low detoxification excretion profile groups.

REVIEW OF LITERATURE

CONJUGATION REACTIONS

It is now generally accepted that there occur in mammals six major conjugation reactions with which they can metabolize and excrete xenobiotics; four are of major concern to humans. These six reactions each have a well-defined role in the metabolism of endogenous compounds and in biosyntheses (12). All involve the participation of a transferase enzyme, which exhibits high specificity for the conjugating agent in question. Each reaction is a biosynthesis, and is energy-requiring. The energy is provided either by the conjugating agent being present in an activated form (most frequently as a nucleotide) or by the prior activation of the xenobiotic substrate (12).

The purpose of the remainder of this chapter is to review the major conjugation reactions, in terms of their substrate versatility, enzymatic mechanism, to discuss briefly upon their biological consequences, and their role in detoxification.

Glucuronic acid conjugation

Glucuronic acid conjugates (glucuronides) represent the most important phase II reaction (6,10). This is so because it is the most versatile of the conjugation reactions in terms of the range of xenobiotic functional groups to which glucuronic acid can attach itself. **Table 1** gives examples of the functional groups and the types of compounds that can react with glucuronic acid to form conjugates. The glucuronic acid residue incorporated into the conjugate is derived from the nucleotide uridine diphosphate glucuronic acid (UDPGA). Therefore, although it is found in abundance and readily available (12,13), concentrations being usually higher in the liver than in any other tissue (14), the concentration of UDPGA has been shown to decrease when the demand for it becomes too high at high substrate dose (15).

The formation of glucuronides involves two steps. First, the synthesis of an

TABLE 1. Types of compounds giving rise to glucuronic acid conjugates (12,14)

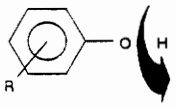
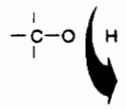
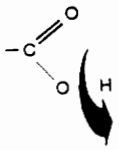
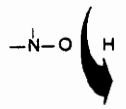
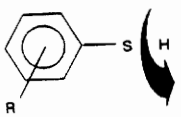
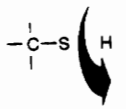
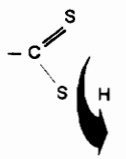
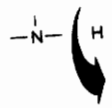
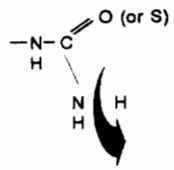
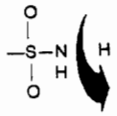
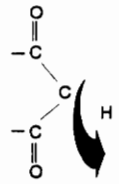
	Substrate Group	Examples
O-GLUCURONIDES		
	phenols	phenol morphine paracetamol
	alcohols enolic alcohols	t-butanol many steroids chloramphenicol 3,5androstadiene, 3,17-dione
	carboxylic acids	bilirubin valproic acid nicotinic acid indole-3-acetic acid
	hydroxylamines hydroxamine acids	N-hydroxy-2-naphthylamine N-hydroxy-2-acetylaminofluorene 4-hydroxy coumarin
S-GLUCURONIDES		
	thiophenols	thiophenol
	thiols	2-mercaptobenzothiazole
	carbamic acids	diethyldithiocarbamic acid

TABLE 1. cont.

N-GLUCURONIDES		
	aromatic amines hydroxylamines	aniline N-hydroxy-2-napthylamine
	tertiary amines	cyproheptadine
	(thio)carbamate	meprobamate
	sulfonamides	sulphadimethoxine
C-GLUCURONIDES		
	1,3-dicarbonyl compounds	sulfinpyrazone phenylbutazone

activated coenzyme uridine-5'-diphospho-D-glucuronic acid occurs, with uridine-5'-diphospho-D-glucose serving as the precursor of the biosynthesis of UDPGA, a highly water-soluble and stable compound (14). There then occurs the subsequent transfer of the glucuronyl group to an accepting substrate. **Figure 1** summarizes the sequence of events involved in glucuronidation.

UDP-glucuronyltransferases (UDPGTs) are membrane-bound microsomal enzymes that exist as a multigene family (EC 2.4.2.17) that results in a range of isoenzymes, each possessing different, but closely related, physical and catalytic properties (14). It is this family of enzymes that carries out the final transfer of the glucuronyl moiety of UDPGA to a variety of acceptor substrates (10,13). The substrate may be both an endogenous compound (e.g., bilirubin, steroid hormones) as well as exogenous xenobiotic compounds (12,13,16,17). Although some isoenzymes appear to be more specific for the conjugation of endogenous compounds, there also appears to be considerable overlap between the conjugation of endogenous and exogenous compounds. Many compounds, which are not themselves substrates, can also be metabolized via phase I metabolism into acceptable substrates for the enzyme. The enzyme is found particularly in the endoplasmic reticulum of the liver, although it is also distributed in the kidney, intestinal mucosa, skin, and adrenal gland, as well as other tissues (17).

The activity of UDPGT in tissues is altered by several substances, such as, drugs, environmental pollutants, and dietary nonnutrients (6,10,18,19). This enhancement of enzyme activity is called induction and is thought to be a consequence of increased denovo synthesis of UDPGT (14,18). Although inducibility appears to be a general property of UDPGT, the degree of induction varies according to tissue and inducing agent (20). Little, however, is known about the inducibility of human UDPGTs, although there is evidence to show that treatment of patients with phenolbarbital-like drugs results in higher UDPGT activity towards 1-naphthol, 4-methylumbelliferone, and bilirubin (21).

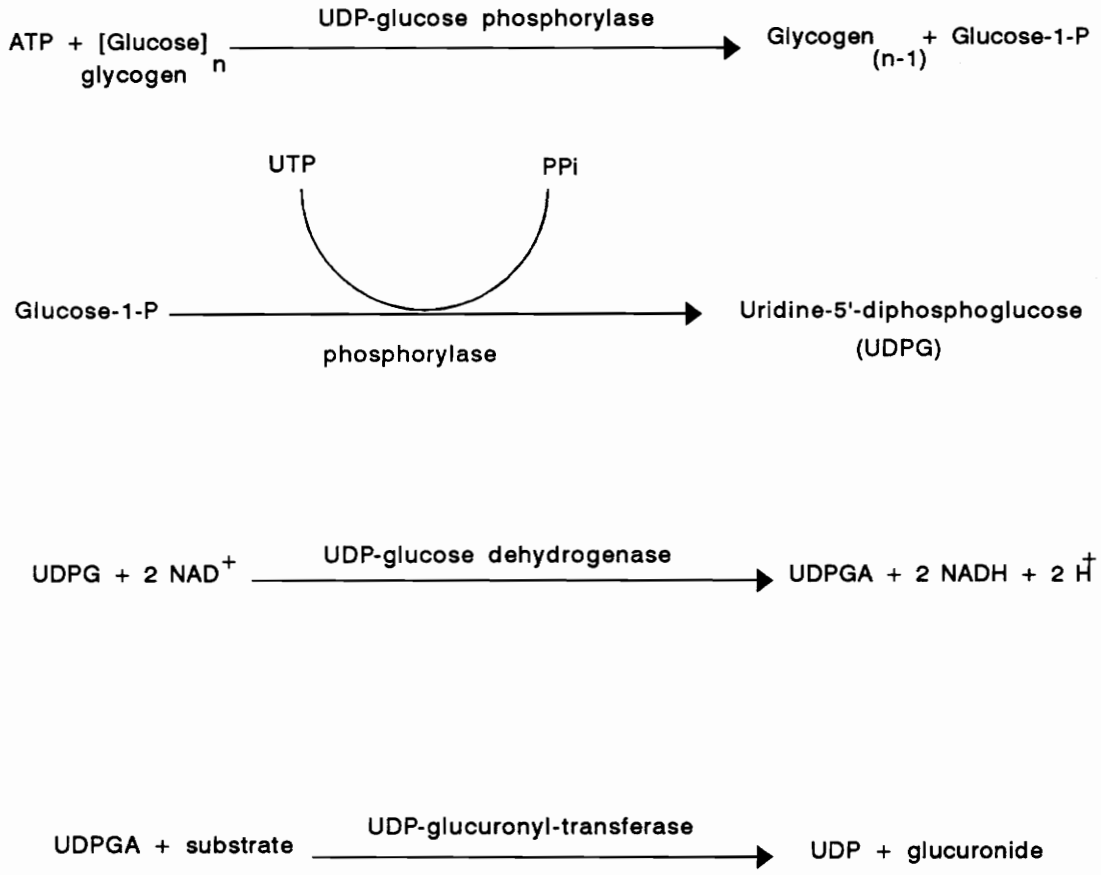


FIG 1. Formation of UDPGA and glucuronides (13)

Virtually all mammals are able to form glucuronic acid conjugates. Yet, there exist certain species, and genetically stable mutants within the species, which are marked by a partial or complete inability to form such conjugates (10,17). Probably the best example of a 'species defect' in glucuronidation is to be found in the domestic cat. The cat is unable to form glucuronides of many compounds which are extensively metabolized via this pathway in most other species (12). In man, a condition known as the Crigler-Najjar Syndrome exists which is due to the complete absence of UDPGT activity (12,13). This may lead to the development of jaundice as a result of insufficient transferase to catalyze formation of bilirubin glucuronide or by drugs competing with bilirubin for glucuronidation at the few active enzyme sites available (10).

Glucuronidation in general profoundly changes the biological effects of compounds as a result of the addition of a large hydrophilic group so that the metabolite will not easily fit into its receptor binding sites. For endogenous compounds (steroid hormones, thyroxine, and similar endogenous alcohols or phenols), glucuronidation seems to mainly play a catabolic role (14). Once glucuronidated, these endogenous compounds are excreted in urine or bile, therefore, glucuronidation is involved in their elimination. It definitely plays an important role in bilirubin elimination. Other glucuronides in the blood are biologically active such as retinoyl ß-glucuronide and glucuronides of 1,25-dihydroxy vitamin D (22,23). Retinoyl ß-glucuronide seems to be an alternative mechanism for the transportation of vitamin A to target tissues, where it is hydrolyzed by ß-glucuronidase to the biologically active form, retinoic acid (23). Thus the glucuronide of these compounds may be the active form of the endogenous compound or a storage form from which the aglycone can be released by ß-glucuronidase.

Sulfate conjugation

Sulfate conjugation represents an alternative to glucuronidation for the

metabolism of a wide range of substrates, although in comparison to glucuronidation, conjugation of drugs and xenobiotics with sulfate occurs less commonly (24). This is credited to the limited pool of sulfate in mammals and to the smaller number of functional groups capable of forming sulfate conjugates (25,26). **Table 2** lists the type of compounds and functional groups that undergo sulfoconjugation.

Sulfation is the actual transference of activated sulfate to an appropriate substrate. This involves three steps which lead to the ultimate formation of sulfate conjugates. These steps are represented in **Figure 2.** There is an initial activation of inorganic sulfate to give adenosine-5'-phosphosulfate (APS). A second activation occurs that forms the coenzyme 3'-phosphoadenosine-5'-phosphosulfate (PAPS), followed by the transfer of the sulfate group from PAPS to the accepting substrate. **Figure 3.** gives an example of conjugation with sulfate to form a sulfoconjugate.

Thus, efficient synthesis of PAPS requires sufficient inorganic sulfate and sulfation of xenobiotics can be limited by the bioavailability of inorganic sulfate (24). The inorganic sulfate necessary for sulfation may be obtained from the diet (with subsequent absorption from the gut), oxidation of sulfur amino acids, and catabolism of sulfated macromolecules (27). *In vivo*, inorganic sulfate in the blood is immediately available for sulfation in the tissues (28).

Because the total pool of sulfate and PAPS is usually quite limited, it can be readily exhausted (12,26,27). If sulfate becomes low, sulfation is almost completely abolished (29) and the only sulfate available arises from sulfoxidation of endogenous L-cysteine as long as it is available (30). Sulfate can be decreased by a diet low in sulfur-containing amino acids or by ingestion of a high dose of a substrate for sulfation such as paracetamol. During periods of reduced sulfate availability, conjugation shifts from sulfation to glucuronidation - the alternative, competing conjugation reaction (12,24,26).

The transfer reaction that leads to the formation of a sulfoconjugate is catalyzed by a number of soluble sulfotransferases present in the cytosol of liver and

TABLE 2. Types of compounds forming sulfates (12)

Functional Group	Example
Primary alcohol	Ethanol
Secondary alcohol	Butan-2-ol
Phenol	Phenol
Catechol	α-methyl-DOPA
Alicylic alcohol	Dehydroepiandrosterone
Heterocylic alcohol	3-Hydroxycoumarin
Hydroxyamine	N-Hydroxy-2-acetoamidofluorine
Aromatic hydroxylamine	2-Naphthylhydroxylamine
N-oxide	Minoxidil

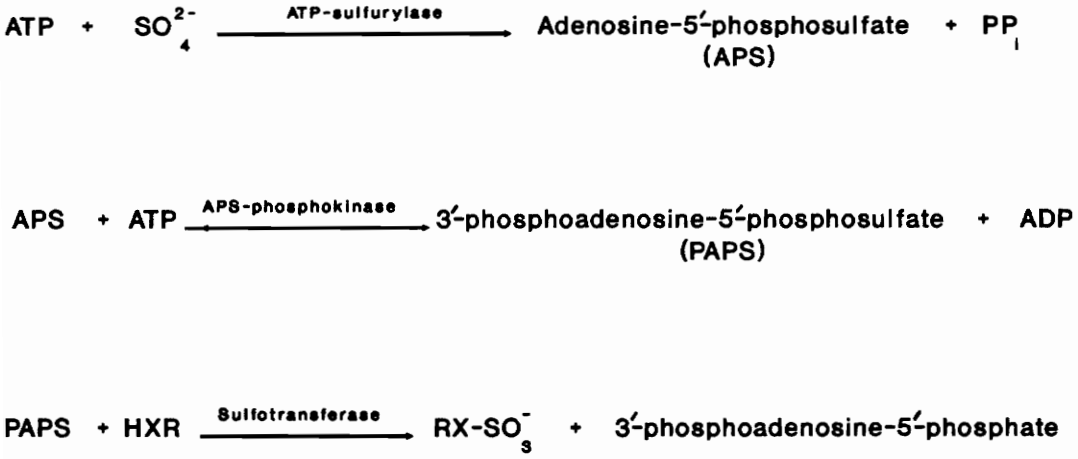


FIG 2. Formation of sulfate conjugates in the cytoplasm (6)

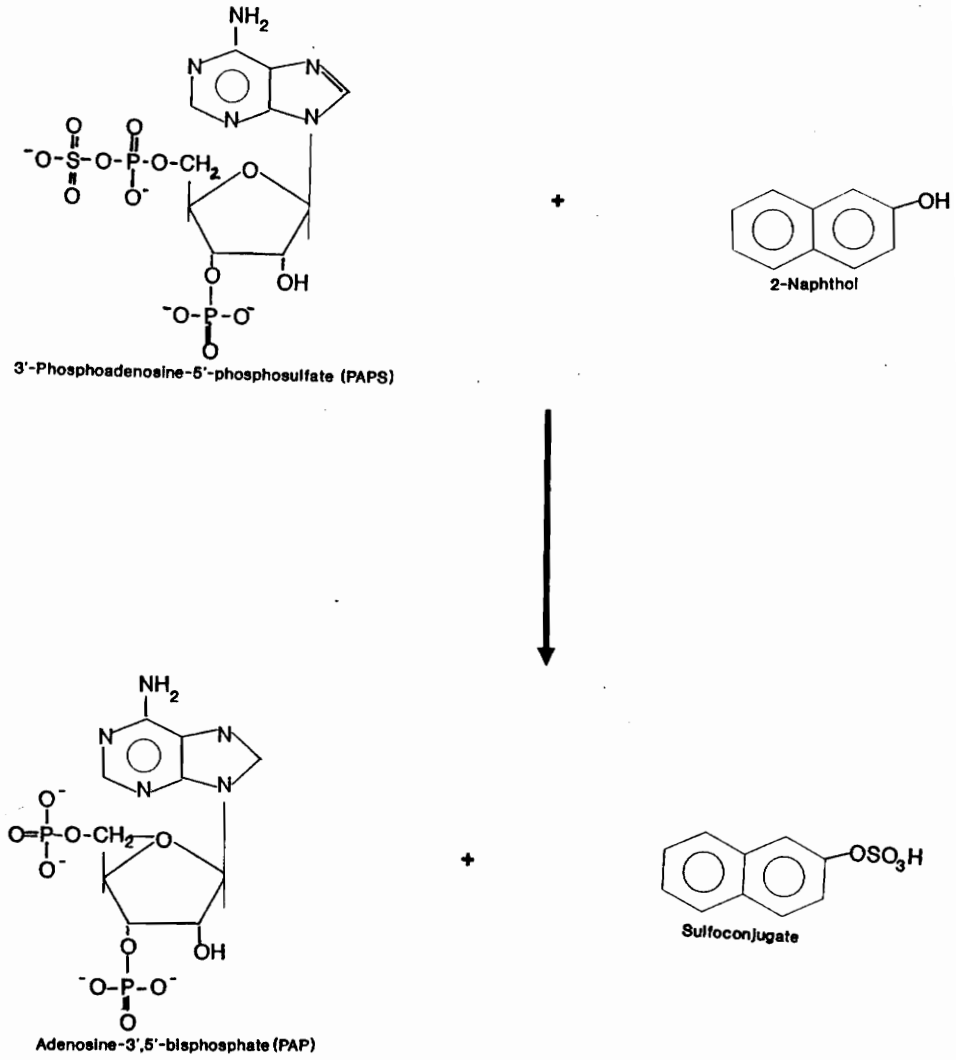


FIG 3. An example of conjugation with sulfate to form a sulfoconjugate (26)

other tissues (6,13,25). The sulfotransferases are a family of enzymes that can be categorized into two groups. The first group conjugates phenolic substrates, and the second group is responsible for steroid conjugation. The two groups can be separated by ammonium sulfate fractionation (31). The phenol sulfotransferase (also known as the aryl sulfotransferase, EC 2.8.2.1.) fraction contains at least four distinct enzyme forms, and is the most important of the enzymes that catalyze the sulfate conjugation of xenobiotics and neurotransmitters (32).

Many low molecular weight, endogenous compounds are sulfated. Their biotransformation usually terminates the biological action of the substrate and leads to excretion of the conjugate in urine (30). It has been estimated that sulfation of catecholamines contributes as much as 10% of the metabolism of dopamine and noradrenaline in the brain (33,34). Sulfation is more important in the metabolism of steroids, being a major conjugation reaction for these compounds (30). Although the sulfates are excreted in urine, there is circumstantial evidence that they may have biological roles as intermediates or storage forms of the parent compound (30). The major form of 25-hydroxy vitamin D_3 circulating in blood is the sulfate conjugate (35). Sulfation also plays an important role in thyroxine metabolism by facilitating deiodination of the hormone leading to rapid and irreversible inactivation (36). Sulfation reduces the toxicity of the bile acids and increases their elimination (30).

Amino Acid conjugation

Many classes of drugs, such as anti-inflammatory, hypolipidemic, diuretic and analgesic agents, are conjugated with amino acids prior to excretion (6). These amino acid conjugations comprise the combination of a carboxylic acid with the amino group of one of a number of amino acids to form a peptide bond (6,12,37). As can be seen from **Table 3**, the particular amino acid used in the conjugation of an acid is a function of the structure of the xenobiotic carboxylic acid, the animal species under consideration, and the bioavailability of that amino acid from

TABLE 3. Amino acids used for conjugation of carboxylic acids (8)

Amino Acid	Species	Acid
Glycine	Mammals (Man)	Aromatic, herterocylic, acrylic acids
	Non-primate mammals	Arylacetic acids
Glutamine	Primates (Man) Rat, rabbit, ferret	2-Naphthylacetic acid
Taurine	Mammals, pigeon	Arylacetic acids
Ornithine	Birds	Aromatic and arylacetic acids
Glutamic acid	Fruit bats	Benzoic acid
Aspartic acid	Rat	o,p'-DDA (DDT metabolite)
Alanine	Mouse, hamster	p,p'-DDA (DDT metabolite)
Histidine	African bats	Benzoic acid

endogenous and dietary sources (6,12,37,38). The major amino acid conjugations involve glycine, glutamine, taurine, and ornithine (12). The most frequently observed amino acid conjugates are those with glycine, which is utilized by the majority of animal species and for the conjugation of a wide variety of carboxylic acids, including aliphatic, aromatic, heteroaromatic and phenylacetic acid derivatives (39). Although conjugation with glycine appears to be the most common, conjugation with glutamine and taurine is also seen in man (2). In mammals, glutamine conjugation appears to be restricted to arylacetic acids, e.g. phenylacetic acid and related compounds (39), while taurine is the most commonly conjugated with bile acids (38). **Figure 4** illustrates the type of organic acids and amino acid that conjugate during phase II metabolism. With a single exception, that of histidine, all the amino acids utilized in conjugation reactions are aliphatic and dietary nonessential (40).

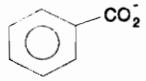
The mechanism of amino acid conjugation differs from the other conjugation mechanisms in a number of ways. First, the amino acid conjugations occur in the mitochondria, whereas, the other conjugation reactions occur in the endoplasmic reticulum or cytosol (2,40). Second, similar to glucuronidation and sulfation, amino acid conjugation requires metabolic activation. In most conjugation reactions, it is the conjugating agent that undergoes activation. However, in amino acid conjugations, it is the carboxylic acid substrate that is activated, in the form of its ester, with coenzyme-A. This activated carboxylic acid then reacts with an amino acid in the presence of an amino acid N-acyl transferase (6,37,40). The sequence of reactions in the conjugation of carboxylic acids with amino acids in shown in **Figure 5**.

The amino acid conjugations are associated, mainly, with the two organs concerned with elimination - the liver and kidney (37,40). The relative importance of these organs in the amino acid conjugations of a compound can vary markedly with species and structure of the carboxylic acid. In some situations, the conjugation may be totally extrahepatic, i.e., renal. In other situations, the conjugation reaction may be both hepatic and extrahepatic. The importance of the two sites is deter-

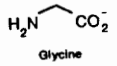
ORGANIC ACID SUBSTRATE

AMINO ACID SUBSTRATE

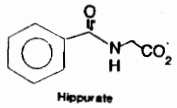
PEPTIDE PRODUCT



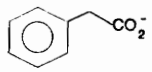
Aryl acid
(Benzoate)



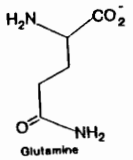
Glycine



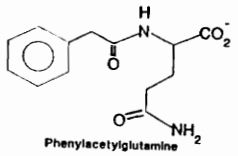
Hippurate



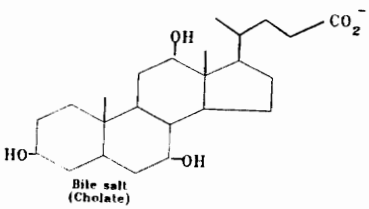
Arylacetic acid
(Phenylacetate)



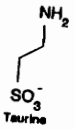
Glutamine



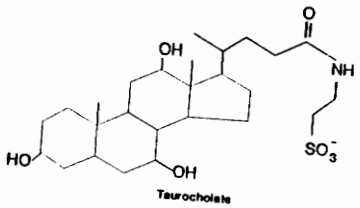
Phenylacetylglutamine



Bile salt
(Cholate)



Taurine



Taurocholate

FIG 4. Examples of conjugation of organic acids with amino acids (26)

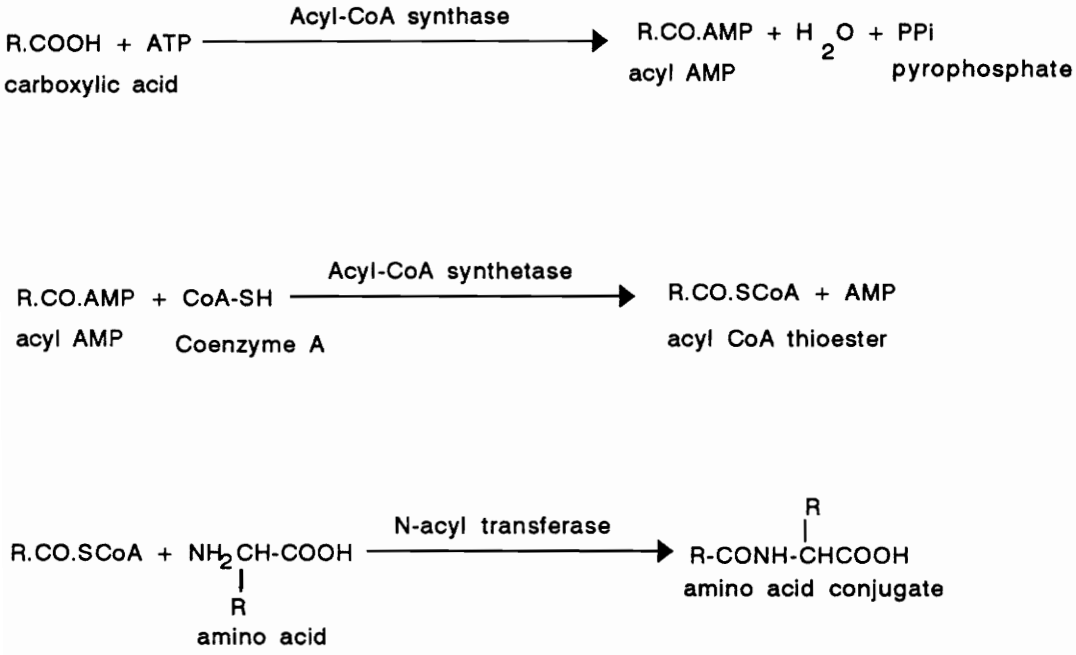


FIG 5. Conjugation of carboxylic acids with amino acids (6)

mined by the species and the particular carboxylic acid undergoing conjugation (37,40).

The two major metabolic options for xenobiotic carboxylic acids involve conjugation with an amino acid (most commonly glycine) or with glucuronic acid. The glycine conjugation mechanism, however, appears to have a relatively limited capacity and is readily saturated by the appropriate substrate (13,37,40). Because of this, the metabolism of a number of carboxylic acids is dose-dependent (13,37). With increasing doses, the importance of the glycine conjugation pathway declines, and a larger, concomitant increase in glucuronic acid conjugation occurs (13). Thus, the amino acid conjugation mechanism appears to be a high-affinity, low-capacity system, whereas glucuronic acid conjugation tends to be a high-capacity, low-affinity system with a broader substrate selectivity. The degree to which glucuronidation formation or amino acid conjugation predominates is also dependent on the animal species and the structure of the carboxylic acid (2,13).

Glutathione conjugation - mercapturic acid formation

Glutathione conjugation is the formation of a thioether link between glutathione and a compound with an electrophilic center. Many xenobiotics, including known carcinogens, are metabolized via the mercapturic acid pathway after conjugation with glutathione. Glutathione (GSH), the tripeptide gamma-L-glutamyl-L-cysteinylglycine, is found in virtually all mammalian tissues and is involved in various metabolic pathways. It is essential in the protection of cells against damage, especially oxidatve damage, induced by various agents, which are potentially harmful electrophilic species, and diseases (13,41,43).

Because of its importance, GSH is present in most cells, although its intracellular concentration varies with the cell type (43). The highest level is found in the liver, presumably necessitated by the specialized role of the liver in the detoxication and excretion of xenobiotics (43). GSH is also found in relatively high

amounts in human erythrocytes probably to prevent oxidative damage due to the oxygen-carrying function of these cells (44).

Xenobiotics conjugate with glutathione at its free SH-group as the unchanged drug or its electrophilic metabo-lite(s) (6). Conjugation with glutathione does not require the initial formation of an activated coenzyme or substrate (6,13,42). The inherent chemical reactivity of the nucleophilic glutathione toward an electrophilic substrate seems to provide sufficient driving force for the reaction to occur in the absence of an enzyme. In this respect, glutathione conjugation differs from the other conjugation pathways. The substrates that undergo conjugation with GSH are enormously varied. The major prerequisite is that the substrate be sufficiently electrophilic. Thus a great number of chemically different classes of compounds can undergo GSH conjugation. However, in terms of general reaction mechanisms, these substrates usually fall into two basic categories: 1) electrophiles that undergo nucleophilic substitution at carbon or a heteroatom, carbons of strained ring systems such as epoxides and ß-lactones are also included and 2) electrophiles with an 'activated' double bond that can undergo addition with GSH (13). **Table 4** summarizes the division of the numerous classes of chemicals into these two basic categories.

Compounds undergoing glutathione conjugation are ultimately found as N-acetylcysteine derivatives or mercapturic acid conjugates (2,6,13). Mercapturic acid formation is initiated by glutathione conjugation, followed by removal of the glutamate moiety by glutathionase. Subsequently, the glycine is removed by a peptidase enzyme, cysteinylglycinase. These two enzymes are found both in the liver and the kidney. In the final step, the amino acid group of cysteine is acetylated by a hepatic N-acetylase resulting in the formation of the mercapturic acid derivative which is readily excreted via the renal system. **Figure 6** depicts a summary of these steps, and **Figure 7** gives an example of glutathione conjugation with ultimate mercapturate formation.

TABLE 4. Two general categories of substrates that conjugate with glutathione (16)

Electrophiles tha Undergo Nucleophilic Substitution by Glutathione		
Site	Class	Example
Saturated carbon atoms	Alkyl halide, sulfates	Methyl iodide Isopropyl methane sulfate
	Nitroalkanes	1-Nitrobutane
	Organophosphates	Methylparathion
	Allyl halide, nitrates, sulfates	Allyl chloride, nitrate, sulfate
	Benzyl halide	Benzyl chloride
	Aralkyl halide	2-Acetamindo-4-chloromethylthiazole
	Aralkyl sulfates	1-Menaphthyl sulfate
Carbon atoms of aromatic/heteroaromatic systems	Halogenated nitrosubstitued quinoline N-oxide	2,4-Dichloronitro-4-nitro-4-nitroquinoline N-oxide
	Nitrofurans	Nitrofural
	Other heterocyclic compounds	Azathioprine
Carbon atoms of strained ring systems	Aliphatic epoxides	Styrene epoxide
	Arene oxides	4-Bromobenzene oxide Naphthalene-1,2-oxide
	ß-Lactones	ß-Propiolactone
Heteroatoms	Organic nitrates	Nitroglycerin
	Organic thiocyanates	Octyl thiocyanate
		Benzyl thiocyanate

TABLE 4 (cont.). Two general categories of substrates that conjugate with glutathione (16)

Electrophiles with 'Activated' Double Bonds that Undergo Nucleophiliic Addition with Glutathione		
α,ß-Unsaturated systems	Ketones	Ethacrynic acid
	Aldehyde	Crotonaldehyde
	Esters	Diethyl maleate
	Nitrile	o-Chlorobenzylidene malononitrile
	Nitro compound	3-(ß-Nitrovinyl)indole
	Sulfone	Methyl vinyl sulfone
Other activated double bonds	Isothiocyanates	Allyl isothiocyanate Benzyl isothiocyanate

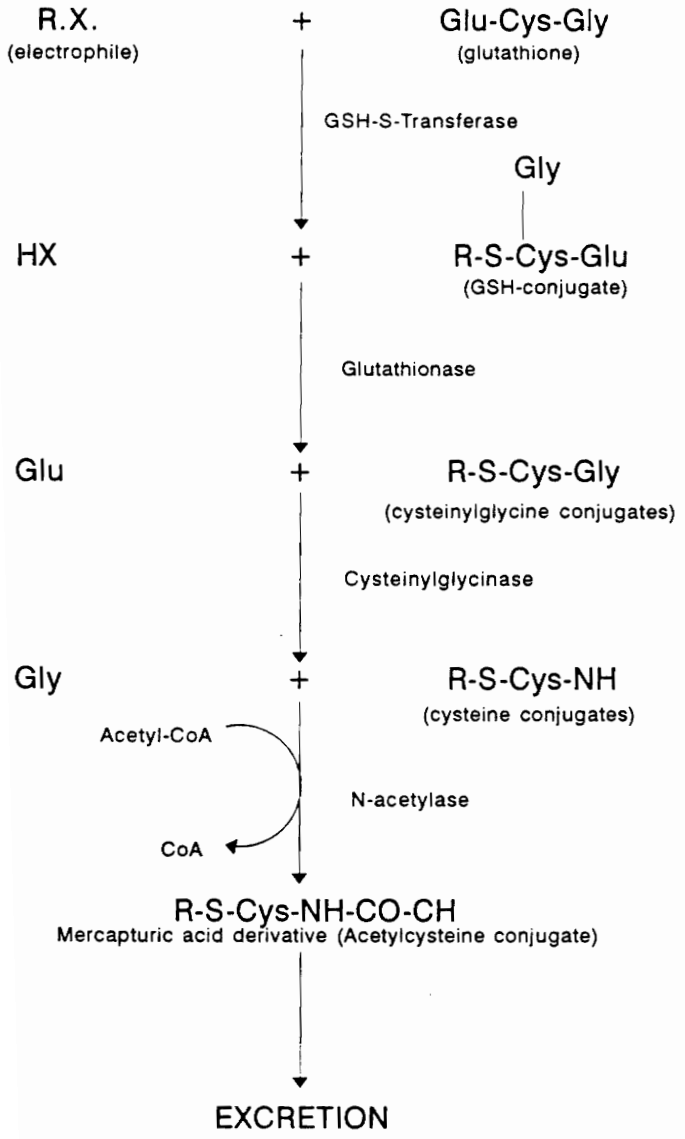


FIG 6. Mercapturic acid biosynthesis (13)

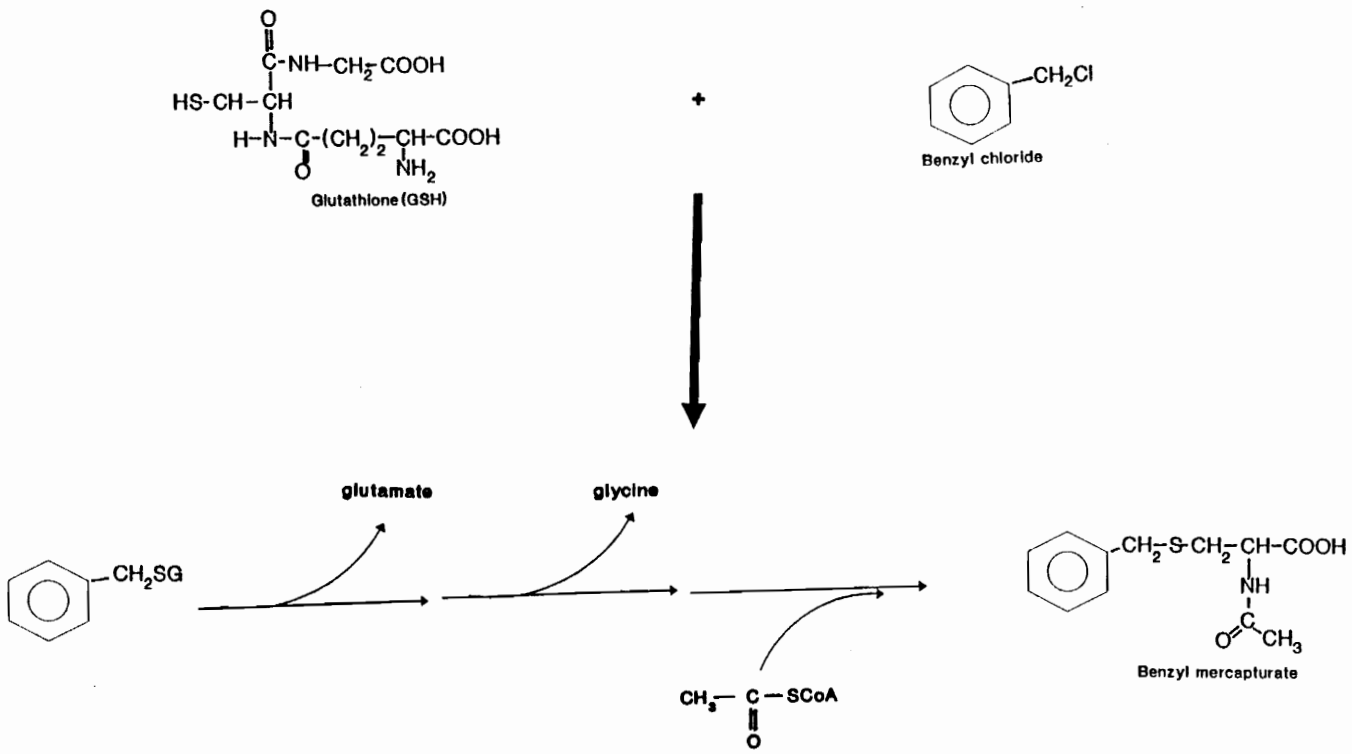


FIG 7. An example of glutathione conjugation and mercapturate formation (26)

The various glutathione conjugations are catalyzed by a family of glutathione S-transferase isozymes located in the cytosol of many tissues (6,12,41). The tissue distribution of the isozymes is a characteristic (both qualitatively and quantitatively) of each tissue and presumably related to the function of that tissue (41). These enzymes exist in multiple forms, and have recently been shown to be dimeric proteins of similar, but nonidentical, subunits (6,12,41). Seven different subunits have been recognized and the various isozymes are homo- or heterodimers of these (45).

Both enzyme induction and depletion of liver glutathione stores contribute considerably to the overall detoxication capacity of GSH conjugation (6,13). The glutathione-S-transferases are inducible by various xenobiotics, such as, phenobarbitone, 3-methylcholanthrene, and stilbene oxide (6,46). These inducing agents can cause increased activity of the GSH-S-transferases by 2- to 8-fold. This increase in enzyme activity is related to an increase in mRNA synthesis, with mRNAs coding for each subunit being affected differently by different inducing agents (6). A diet lacking protein, and hence cysteine and methionine, results in a GSH deficiency. This becomes very apparent if there happens to be a high demand for GSH conjugation due to administration of a high dose of a substrate that is conjugated via GSH (47).

The liver is an important organ of biotransformation and, appropriately, hepatic GSH levels are relatively high - about 170 mg/100 g in rat liver (42). Depletion of liver GSH stores, due to increased doses of xenobiotics, increases the susceptibility of animals to the hepatotoxicity of certain compounds (13). In addition, the severity of the hepatic necrosis is increased with increasing GSH depletion (13).

Thus, in conclusion, the GSH conjugation mechanism can be considered to be of great significance in protecting the body against the harmful effects of electrophiles and radical species. Additionally, it facilitates the excretion of many xenobiotics, since most of the products of the catabolism of conjugates are highly

polar and water soluble.

Summary

It is evident, from the previous discussion, that the conjugation reactions are of great importance in the metabolic disposition of xenobiotics of all types in the animal body. They have been increasingly recognized as having pharmacological and toxicological implications. Any attempt to relate together the chemical structure, metabolic fate and biological actions of a chemical must take conjugative metabolism into account (12).

The conjugation reactions constitute the second part of the biphasic sequence of xenobiotic metabolism in the body. A balance between the phase I and phase II enzymes largely determines the disposition to drug toxicity. It is the outcome of this balance which leads to efficient detoxification or the accumulation of toxic metabolites.

By using endogenous conjugation agents, which have well-defined roles in biosynthesis and intermediary metabolism, the conjugation reactions represent an interface between the metabolism of xenobiotics and the biochemistry of endogenous compounds (12). The limited supply of certain conjugating agents underlies the ready saturability of some of the conjugating reactions.

INTERINDIVIDUAL AND INTRAINDIVIDUAL VARIABILITY

Increasing evidence has accumulated that many internal and external factors may profoundly influence xenobiotic metabolism and excretion. This should not be surprising, in that, all or most of the reactions involved in detoxification are biochemically complex. Therefore, there is much opportunity for biological variation to occur of both heritable and environmental origin. The many factors that affect the rate of metabolism of xenobiotics in people are divided, by Gibson and Skett (6), into two major categories - internal factors (genetic, sex, hormones, etc.) and external

factors (diet, environment).

There is wide variability among individuals in their response to the same dose of various drugs. Much of this interindividual (between subject) variability in drug response can be explained by pharmacokinetic factors, e.g., absorption, distribution, metabolism and excretion (48). The rate at which these processes occur is influenced by many factors. Some of the variability can also be explained by genetic aspects because they determine the ultimate difference between individuals. This individuality in metabolism and capacity to eliminate drugs also extends to environmental xenobiotics, which are metabolized by the same enzyme systems.

Interindividual response to drug/xenobiotic exposure is well recognized and better understood than intraindividual variability. Intraindividual (within subjects) variability may be defined as the variability in the value of a measured parameter, within an individual, when assessed on different occasions (49). The degree of variability within the same individual can be markedly different from the variability within another individual even though environmental perturbations may be equivalent (49). When studies were conducted to determine the elimination half-life of lincomycin, the ranges of elimination between subjects varied by as much as 3-fold (49). Similar results were found with other drugs (53).

The relationship between intraindividual and interindividual variation needs to be noted because, often times, the former is either ignored or assumed to be negligible, when compared to the latter, during scientific inquiries. As indicated the magnitude of intraindividual variation is not always small. The more subjects are environmentally perturbed, the larger is the magnitude of intraindividual variability relative to interindividual variability, although the former can never exceed the latter (50).

Although internal factors (i.e. genetics, hormones, etc.) are more likely the cause of the intervariability, external factors, such as age, disease, drugs, diet, and environment, can and do contribute to interindividual differences and also to in-

traindividual variability (**Figure 8**)(49). Depending on the population, the specific xenobiotic being examined, and the particular factor being studied, the magnitude of these variations can vary greatly (49-53). Thus it becomes obvious that the control of drug/xenobiotic metabolism is an extremely complex system with many factors influencing the rate of metabolism. Some of these factors may also have an interactive role in drug/xenobiotic metabolism. The following chapters will try to highlight the principal factors that affect drug metabolism and, therefore, need to be considered when trying to determine detoxification abilities of individuals.

VARIATION DUE TO GENETICS

It seems obvious that the ultimate difference between individuals (interindividual variability) is due to genetics. There is evidence to suggest that the preponderance of the difference between individuals in the handling of xenobiotic compounds is due to genetic variability in the biotransformation reactions (54,55). In man the possibility of showing a pure genetic influence on drug (xenobiotic) metabolism is hampered by interfering influences from environmental sources. It has, however, been possible to show probable genetic effects on drug (xenobiotic) metabolism.

The significance of genetic influence on drug (xenobiotic) metabolism varies according to the type of drug and metabolic pathway concerned (6). Results from studies of twins (56), traditionally useful in elucidating the relative contributions of nature and nurture, demonstrate that interindividual pharmacokinetic variations disappear within monozygous twins but are preserved within dizygotic twins. Twin studies on the kinetics of almost a dozen drugs have been performed in different laboratories located in four countries to compare the relative contribution of genetic and environmental factors to large interindividual variations in drug disposition (55-58). Results from all the studies were similar. Variation virtually disappears within monozygotic twins, but is preserved within most dizygotic twins, where the variation is almost as much as the general population. Thus, genetic factors can be predomi-

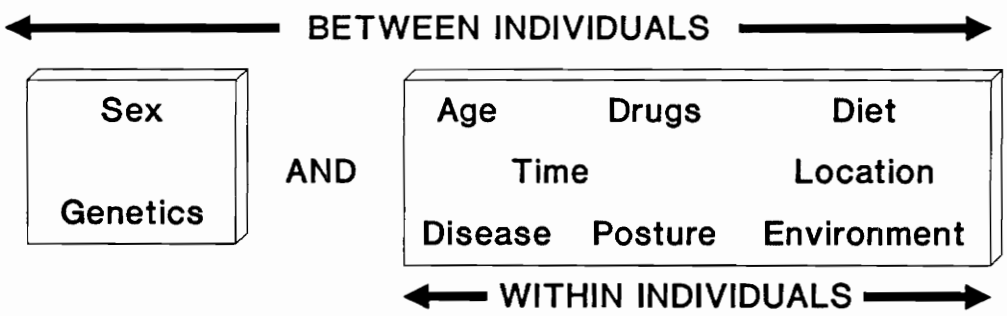


FIG 8. Sources of variability (49)

nant determinants for observed variations between individuals.

A few drug-metabolic reactions are predominantly under genetic control and exhibit polymorphism in the population. Interindividual variation in the ability to hydroxylate debrisoquine (an adrenergic blocking agent) and sparteine (an antiarrhythmic and oxytoxic agent) are examples (59-62). Two phenotypes, poor metabolizer (PM) and extensive metabolizer (EM), have been established by measuring the urinary excretion of the parent compound and its metabolites over a 12-h period after an oral administration of a single dose of the parent compound. A metabolic ratio (amount of drug to amount of metabolites in urine) is then determined for each subject, and is assumed to represent the metabolizing capacity of that subject (61). When these metabolic ratios are plotted in form of a frequency distribution histogram (**Figure 9**), a clearly discontinuous shape can be observed (62). By definition, the subjects in the lower mode represent the extensive metabolizer phenotypes, whereas, the hydroxylation deficient subjects (poor metabolizers) are located in the upper mode of the frequency distribution histogram (62).

Although numerous studies have looked at variations in the ability to oxidize drugs and the factors responsible for such variation, little work has been done to determine the extent and nature of the interindividual variations that may occur in the various conjugation reactions. This may be due to the perception of the conjugation reactions as secondary in importance to the phase I reactions (16, 63).

Evidence for species variations in phase II reactions exists and can be more dramatic, primarily because of the presence or almost complete lack of certain enzymes involved in these conjugation reactions (64). The most common example being the complete absence of glucuronidation in the cat. A human disorder, congenital familial nonhemolytic jaundice, is associated with decreased glucuronyl transferase activity in the conjugation of bilirubin and some drugs (64).

One of the best documented examples of a genetically determined conjugation reaction is the acetylation of isoniazid (65-66). Isoniazid is normally cleared from

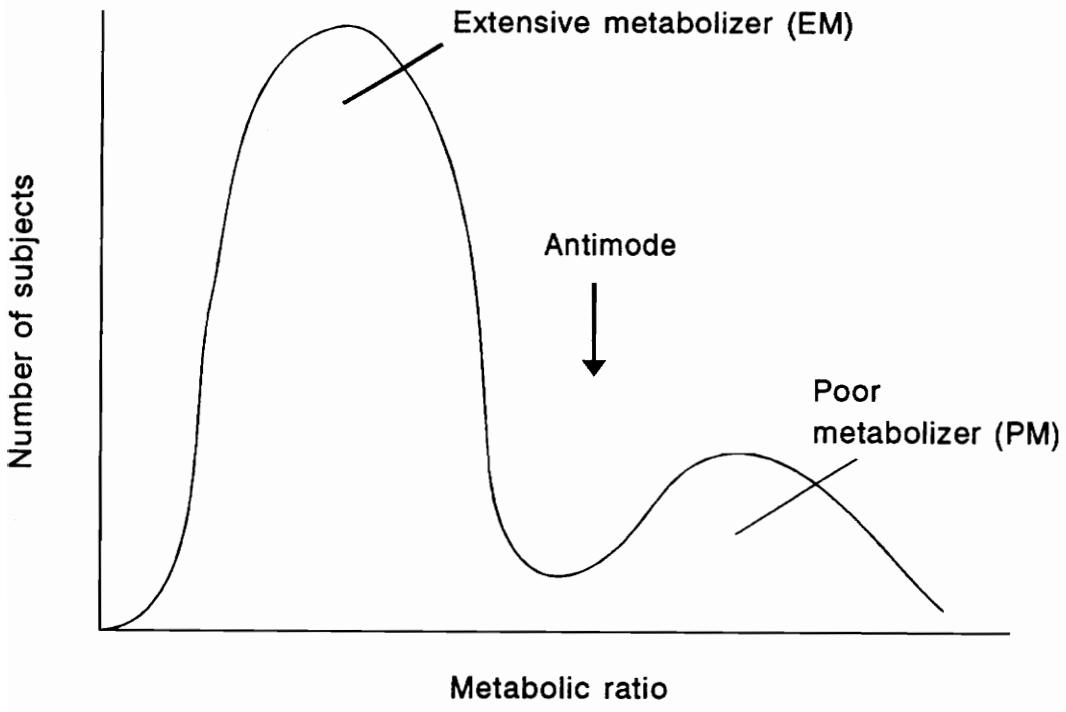


FIG 9. Drug metabolic ratios in form of a frequency distribution histogram indicating polymorphic drug hydroxylation in man (62)

the body by N-acetylation. The acetylator phenotypes, 'fast' and 'slow' acetylators, are related to the rate of metabolism and clearance of isoniazid and its metabolites. In a 'slow' acetylator, the therapeutic dose of the drug is sufficient to cause marked build-up of unchanged drug, and can cause the toxic side-effects of central stimulation and peripheral neuritis. 'Fast' acetylators are, however, more susceptible to drug-induced hepatic damage. Genetic investigation revealed that this characteristic is controlled via two autosomal alleles, R for 'fast' acetylation and r for 'slow' (6).

Caldwell and Davies (67), using the probe drugs paracetamol and salicylamide, investigated the interindividual variation of sulfation and glucuronidation. A total of 62 healthy, young medical students and laboratory staff were given 1 g paracetamol or salicylamide, with 100 ml water, after a light breakfast. Urine samples were collected for 0-8 hours and analyzed for conjugates. A frequency distribution of the ratio of the % total recovery as the glucuronide and the % recovery as the sulfate (G/S ratio) showed that the distribution of the metabolic variation (for paracetamol), in this panel of volunteers, deviated markedly from normality. Insufficient data were available to determine whether or not it was a bimodal distribution. The frequency distribution for salicylamide, again expressed as G/S ratio, was found to have a normal distribution. Thus, despite the close structual similarities between these two drugs, their metabolic patterns are apparently under separate control. For both drugs, certain individuals had a reduced ability to form the respective sulfate conjugates, and in the case of paracetamol, this deficiency was so marked as to lead to a nonnormal distribution of the G/S ratio.

Although there is evidence to indicate that genetic factors have a great influence on an individual's ability to metabolize drugs/xenobiotics, it has been found that environmental factors can greatly modify this 'genetic factor'.

VARIATION DUE TO THE ENVIRONMENT

In addition to genetic influence, the effectiveness of the detoxication pathways

is simultaneously affected by a multitude of environmental factors. It has become increasingly evident that environmental factors other than atmospheric and water pollution influence metabolism and response to drugs (68). Such other influences include diet, smoking, medications, disease, fever, ingestion of alcohol, and exposure to environmental chemicals (54). Studies continue to shown that the environment around an individual prior to, during, or after exposure to drugs/xenobiotics, has a significant impact on the rate of the metabolism of these compounds (50).

Awani et al. (69) determined the inter- and intraindividual variability in the pharmacokinetics of labetalol, an antihypertensive agent. Each subject received, orally, five different formulations of labetalol containing 100 or 200 mg of the drug in a randomized cross-over design on five separate occasions. A 7-day washout period separated each phase of the study. The ratio of intraindividual to interindividual variability was used as an index of the relative contribution of environmental and genetic factors to the variability in the pharmacokinetic parameters. Large interindividual variability was found. Also, considerable interindividual variability in labetalol half-life was found. Therefore, the investigators concluded that environmental factors must be having a great influence on labetalol disposition. This would indicate that environmental factors can alter the genetically determined rate at which a particular subject eliminates drugs and environmental chemicals.

Large intraindividual variations in the plasma half-life of several drugs have been attributed to the influence of these environmental factors (50,51,70). Alvares et al. (51) determined the magnitude of intraindividual variation by measuring the plasma elimination rates of phenacetin in seven subjects on five occasions at 6-wk intervals for each subject. No attempt was made to control the lifestyles or diets of the subjects during the course of the investigation. Although it is difficult to observe genetic and environmental influences in a completely isolated manner, the investigators felt that repeated observations on the same subject would provide the most feasible means of controlling for the effects of genetic factors in unrelated

individuals and of assessing variability in response to environmental perturbations. **Figure 10** illustrates the intraindividual variability measured in the seven subjects. The large variations observed were felt to be due to a variety of environmental factors, such as, dietary changes, stress, hormonal changes, seasonal changes, etc. If subjects are kept under near basal conditions, i.e., subjects are minimally, rather than moderately or heavily, exposed to environmental inducing or inhibiting agents, then the intrasubject variability can be as small as 10% of the mean value for each subject (50).

A few studies have looked at how conjugation reactions (phase II) might be influenced by environmental factors (52, 71-75). Dollery et al. (52) studied antipyrine and paracetamol metabolism in a free-living population of 147 employees of four large London firms. Subjects were given 1.5 g of paracetamol with 150 ml of drinking water after an overnight fast. Saliva samples were taken at 2, 3, 5, 8, 24, and 32-h following this dose. The variance of the total body clearance of paracetamol was analyzed in relation to nutritional indices, social drug usage, cigarette smoking, alcohol intake, and coffee and tea intake. The environmental factors studied, especially cigarette smoking and coffee and tea consumption, appeared to exert less influence upon the metabolic clearance of paracetamol than upon that of antipyrine. This suggests that the conjugation reactions may be less affected by environmental factors than are oxidation reactions.

More recently in our laboratories (71-74), the daily excretion of glucuronides, sulfates, and amino acid (glutamine, glycine, and taurine) conjugates in a free living population of adult males was determined. For all three pathways, large intra- and interindividual differences were observed. This large variability probably masked any effects of diet, environment, or genetics since no significant correlations on excretions were observed relative to such parameters as charbroiled food intake; the use of alcohol, caffeine, tobacco, and marijuana; and the incidence of cancer in the families of the subjects.

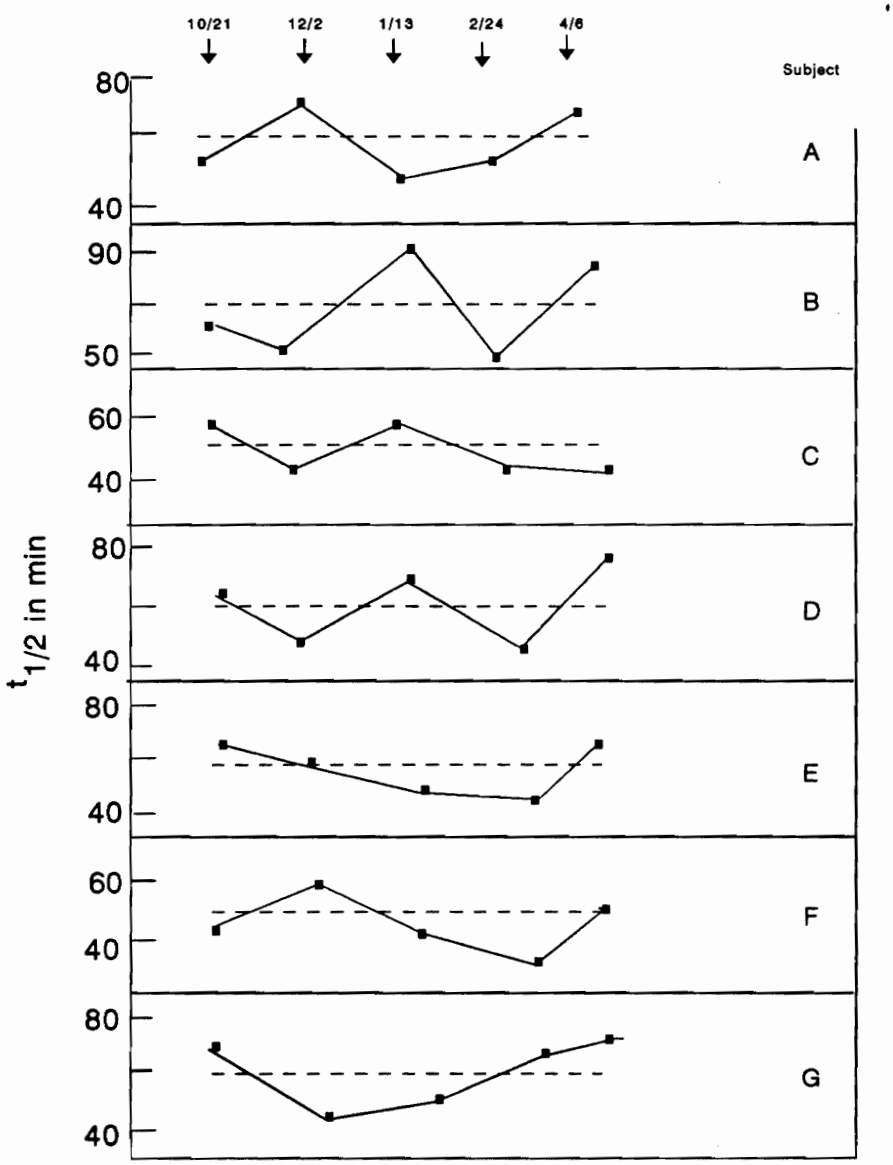


FIG 10. Intraindividual variation in plasma phenacetin half-live in seven normal subjects. Phenacetin (900mg) was given orally to each subject at 6-wk intervals indicated at the top of the figure (51).

As has been seen, there are numerous factors that can affect the way in which the body handles drugs/xenobiotics, varying from the genetic make-up of the person to how much tea or coffee they drink. The influence of environmental factors, on top of obvious genetic differences, leads to the interindividual differences seen in the general population. Also, the ability to metabolize drugs/xenobiotics is very dynamic, even within the same subject.

EFFECT OF DIET ON DRUG METABOLISM AND CONJUGATION

One of the factors which most influences metabolism and the disposition of exogenous chemicals is nutrition (14,76-79). Nutrition influences the biotransformation reactions and the outcome of competition between conjugation reactions in two major ways: (1) by supplying the nutrients that are required to synthesize enzymes, cofactors, etc., in both the phase I and phase II reactions and (2) by the presence of contaminants and natural xenobiotics, or nonnutrients in the food, which are strong inducers or inhibitors of the enzymes of the biotransformation reactions.

Both phase I and phase II reactions are usually catalyzed by enzymes. Because proteins are required for the synthesis of these enzymes, any nutritional state which reduces the availability of amino acids could be expected to reduce the amounts of xenobiotic metabolizing enzymes (76). This can occur when the caloric intake is low, since under these conditions protein will be catabolized and used as a source of energy reducing the availability of amino acids for enzyme synthesis (77,78). Other nutrients are also an integral part of the biotransformation reactions. **Table 5** and **6** lists the other nutrients and their role(s) in the phase I and phase II reactions (77).

A deficiency in the diet of a precursor for a cosubstrate for the conjugation reactions may lead to toxicity or a difference in the types of conjugates excreted (14). Nutrition is especially important for sulfur-containing cosubstrates like glutathione, PAPS, taurine, and indirectly, S-adenosylmethionine because their synthesis depends

TABLE 5. Nutrients in phase I oxidation reactions (2,5)

Nutrients	Component of oxidizing system requiring nutrient
Protein	Apoenzymes & membrane
Lipid	Membranes
Nicotinic acid	NADPH
Riboflavin	FMN & FAD in NADPH-cytochrome c reductase
Glycine	Heme (in Cyt P450)
Pantothenic acid	CoA (ALA synthesis)
Fe	Heme
Cu	Ferrochelatase in heme synthesis
Ca	Maintenance of membranes
Zn	Maintenance of membranes
Mg	Maintenance of membranes
Mg	Maintenance of membranes
Vitamin A	Maintenance of membranes
Vitamin E	Maintenance of membranes

TABLE 6. Nutrients Utilized in Phase II Reactions (59)

NUTRIENT	USE	PHASE II REACTION
Carbohydrates		Glucuronide synthesis
Glucose	Synthesis of UDPGA, N-acetylglucosamine, ribose, glucose	Rate conjugations
Proteins and derivatives		
Glycine	Conjugating agent	Hippuric acid synthesis
Glutamine	Conjugating agent	Glutamine conjugation (man and monkeys)
Glutathione	Conjugating agent	Mercapturic acid synthesis
Cysteine and cystine	Source of S for thiosulfate and sulfate; source of taurine	Cyanide detoxication; sulfate conjugation; taurine conjugateion
Methionine	Synthesis of adenosylmethionine	Methylation
Ornithine	Conjugating agent	Ornithuric acid synthesis (birds and reptiles)
Taurine Serine Aspartic acid Arginine Alanine Glutamic acid Certain peptides	Rare conjugating agents	Conjugations found occasionally in certain species with specific compounds
Tryptophan	Source of formylkynurenine	Formylation (a rare reaction)
Fats		Acetylation
Acetyl (can also be derived from carbohydrate and protein)	Synthesis of acetyl-CoA	
Vitamins		
Panothenic acid	Synthesis of CoA	Hippuric acid synthesis, glutamine conjugation; acetylation
Lipoic acid	Synthesis of aroyl-CoA and arylacetyl CoA	Hippuric acid synthesis, glutamine conjugation
Folic acid	Synthesis of 5-methyltetrahydrofolic acid	Certain methylations, e.g. of dopamine
	Methyl-B-12?	Certain methylations, e.g. of Hg?
Vitamin B-12	Hydroxocobalamin	Cyanide detoxication
Nicotinic acid	For DPN needed for many enzymes, e.g. synthesis of UDPGA	Glucuronide synthesis

on the supply of L-cysteine (14). If glutathione is limiting, it may affect the ability of an individual to defend himself against potentially toxic metabolites such as reactive intermediates. If PAPS is limiting, conjugation may shift from sulfation to glucuronidation causing a change in the excretion profile of conjugates. Thus, it is clear that all the reactions of xenobiotic metabolism are dependent upon the nutrient supply of the body.

Diet should be considered a highly complex chemical mixture which includes such variables as (a) preformed exogenous carcinogens, e.g., aflatoxin and pyrolysates; (b) carcinogenic precursors, e.g., secondary amines and nitrates; (c) type and relative proportion of nutrients, e.g., ratio of saturated to unsaturated fats and trace elements, etc., (d) nonnutrients, e.g., goitrogens, steroids, antibiotics, etc.; and (e) nonspecific effects, e.g., calorie intakes (76). Therefore, eating must be regarded as one of the most hazardous activities of the mammal. Mammals may be exposed to naturally occurring and synthetic chemicals, that are toxic or carcinogenic, via ingestion of these compounds along with their food. This common route of exposure to both nutrients and toxins raises the obvious questions of whether the response to ingested toxins may be modified by the prior, concurrent, or subsequent ingestion of specific nutrients and nonnutrients.

Several researchers have found that dietary protein, carbohydrate, cruciferous vegetables, and charcoal-broiled beef can markedly alter the patterns of chemical biotransformation in normal subjects (79-88). The effects of the isocaloric substitution of carbohydrate for protein on the metabolism of antipyrine and theophylline was studied in six male subjects. During this time subjects were kept on a controlled diet for a period of 4-wks (81-83). A high protein diet was achieved in this study by including protein-rich foods such as meats, fish, cottage cheese, egg whites, and a liquid dietary supplement. Foods rich in carbohydrate, such as, baked goods, cereal, candy, fruit, and potatoes were included in the high carbohydrate diet. The change from a high-protein to a high-carbohydrate diet resulted in a decrease

in protein intake from 44 to 10% of total calories and an increase in carbohydrate from 35 to 70%, while fat remained constant at 20-21%. A single dose of each test drug was given, in the fasting state and food was withheld for 2-h, on days 10 and 14. During this time, mean plasma half-lives ($t_{1/2}$) and metabolic clearance rates (MCRs) of both drugs were determined. The $t_{1/2}$,s of both drugs were substantially shorter and the MCRs were greater during the high-protein intake than during the high-carbohydrate period (**Fig. 11**). This would indicate that the drugs were metabolized more rapidly during the high-protein dietary period than during the high-carbohydrate dietary period.

The effects of substituting fat for carbohydrate or protein was also investigated (82). The average $t_{1/2}$'s for antipyrine and theophylline were found to be longer during the high-carbohydrate, as well as the high-fat dietary periods, than during the high-protein diet period (**Fig. 12**). Changes in $t_{1/2}$ were accompanied by reciprocal changes in MCR's. There was also a small, but statistically significant, decrease in the average $t_{1/2}$ for antipyrine during the high-fat diet as compared with the high carbohydrate diet period. Thus, from the results of this study, it can be concluded that a substitution of dietary protein for either carbohydrate or fat can result in an acceleration of drug (xenobiotic) metabolism, whereas, substituting fat for carbohydrate has little, or no, effect on drug (xenobiotic) metabolism rates.

Vegetables contain a variety of natural chemicals whose effect on specific metabolic systems, such as those involved in the oxidation and conjugation of foreign chemicals, are not well known. This fact prompted researchers to study the effects of such vegetables, specifically cabbage and brussels sprouts, on drug metabolism in humans (86). Ten, normal subjects were fed 1) a control diet for 10 days, 2) the same control diet for three more days, followed by a diet containing brussels sprouts and cabbage for seven days, and 3) a return to the control diet for 10 days. Antipyrine and phenacetin dispositions were studied on days 7 and 10 respectively of each dietary period. For antipyrine, a small but statistically significant decrease in

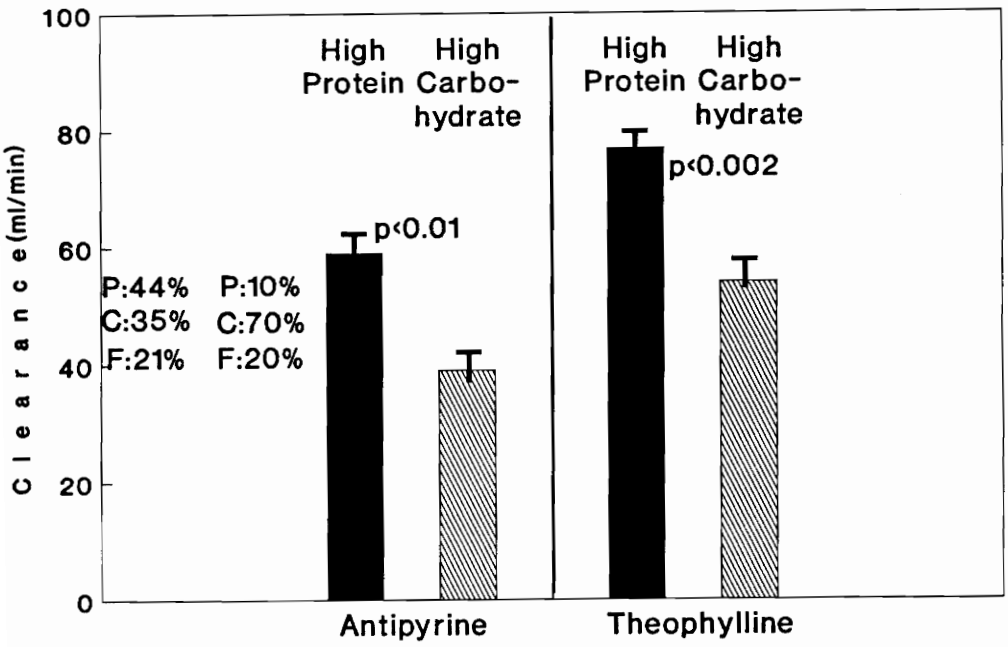


FIG 11. Effects of exchanging protein and carbohydrate in the diet on drug metabolism in six normal subjects. A high protein diet (44% of total calories as protein) was fed for two weeks, followed by a high (70%) carbohydrate diet for two weeks. Half-lives (mean + SE) are shown (82)

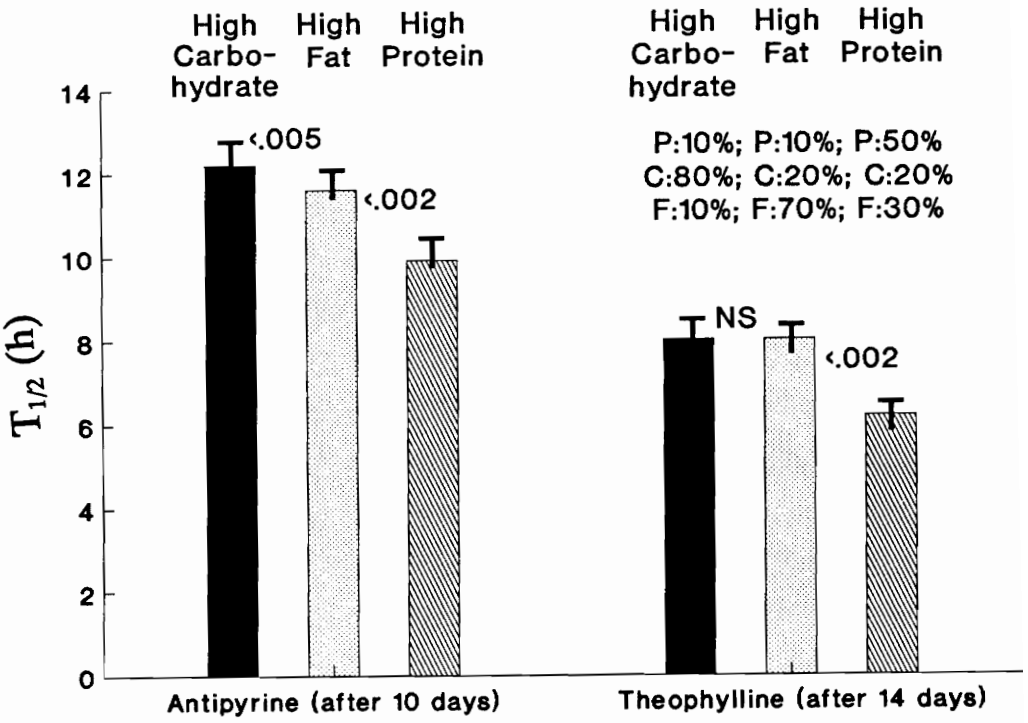


FIG 12. Effects of high protein, high fat, high carbohydrate diets on antipyrine and theophylline metabolism in six subjects. Each diet (composition shown) was fed for two weeks and antipyrine and theophylline metabolism was studied on days ten and 14, respectively, of each dietary period. Half-lives (mean + SE) are shown (82)

the mean $t_{1/2}$ (13%) and an increase in the MCR (11%) indicated a stimulatory effect of these vegetables on the metabolism of the drug. The MCR for phenacetin was increased by 34-67% during the vegetable diet period, indicating that the metabolism of phenacetin was also stimulated by these vegetables.

Cooking at high temperatures can produce chemical changes in foods, especially when conditions exist that favor the formation of products of incomplete combustion (86). Therefore, it is not surprising that the experimental evidence indicates that charcoal-broiled beef accelerated the metabolism of drugs, such as, phenacetin, antipyrine, and theophylline in man (82).

Most of the research dealing with the effect of diet on drug (xenobiotic) metabolism has focused on phase I reactions. Few researchers have looked at the effect of diet on phase II, or conjugation, reactions. Pantuck et al. (87) studied the effects of cruciferous vegetables, specifically cabbage and brussels sprouts, on drug metabolism of oxazepam and acetaminophen in humans. These two drugs were selected because oxazepam, in man, is almost exclusively metabolized by conjugation. Acetaminophen is metabolized primarily by conjugation, the glucuronide and sulfate conjugates being the urinary excretion products. Oxazepam and acetaminophen metabolism was measured in 10 normal men after they had been fed 1) a control diet, 2) a cabbage- and brussels sprouts-containing diet, and 3) the control diet a second time. The control and test diets were identical in every respect except for vegetable content. Each diet period lasted for 10 days with oxazepam being taken on day 7 and acetaminophen on day 10. The test diet stimulated metabolism of acetaminophen via enhanced glucuronide formation. Results for oxazepam metabolism were not as conclusive. The authors concluded that the presence of brussels sprouts and cabbage in the diet can enhance glucuronide conjugation in man.

Because of the relationship between toxicology and nutrition, Truhaut and Ferrando (88) recommended that, when dealing with toxicological experiments, it is

advisable to account for nutritional causes capable of altering the body reactions to toxic substances. Thus, Abbott et al. (89) studied the effect of a purified diet vs. a commercial crude diet on the components of the hepatic mono-oxygenase system of rats. Little or no consistent diet-related difference was observed, nor were results significantly less variable with one or the other diet. These results differ from studies dealing with humans and the effect of diet on xenobiotic metabolism. The discrepancy may be due to the fact that the human diet is so much more variable than any laboratory diet that would be given to lab animals and, therefore, a greater difference would be seen between a crude diet and a semi-synthetic diet and their effect on drug metabolism.

In conclusion, it becomes obvious that there must exist appreciable inter- and intraindividual variations in drug (xenobiotic) metabolism rates which reflect both internal (genetic, hormonal, etc.) and external (diet, etc.) factors. The diet, which represents the major source of exposure to foreign chemicals in humans, probably accounts for a considerable portion of this variability (6).

ASSESSMENT OF DETOXICATION ABILITY IN MAN

Given that xenobiotic metabolism is highly complex and variable, how can an individual's ability to detoxify be determined? The principal methods in use for the assessment of detoxification capability include the direct measurement of enzyme activity *in vitro*, determination of the pharmacokinetics of model compounds, and the observation of changes in the disposition of an endogenous substance (reviewed by Park (86); Park (90)).

Theoretically, measurement of enzyme activity *in vitro* is the ideal method for the assessment of an individual's detoxifying capacity. However, this method is limited by both practical and ethical considerations (90). Liver biopsy samples are usually only taken when liver disease is suspected. Tissue surplus is made available for drug metabolism studies after histological requirements are met. If the liver

sample proves to be histologically normal, then enzyme rates can be related to such factors as, diet, drug influence on enzyme rates, smoking habits, etc. (91,92).

When linked to parallel *in vivo* studies, this method of detoxification capacity assessment can be a very powerful research tool. A number of groups have investigated the relationship between drug metabolism *in vivo* and *in vitro* in the same subjects. Back et al. (92) studied the *in vivo* and *in vitro* effects of the antimalarial drugs chloroquine and primaquine on rat liver. These researchers found that there was a good correlation between *in vivo* and *in vitro* enzyme rates, in that both chloroquine and primaquine demonstrated inhibited enzyme activity regardless of which method was used. Although the direct measurement of enzyme activity *in vitro* can be a useful technique towards the determination of an individual's capacity to detoxify, especially when coupled with *in vivo* studies, it is an invasive procedure and therefore not practical. Also, the premedication required to anesthetize the patients may have an influence on the hepatic drug metabolizing enzymes and, thereby, confound any results obtained.

The most widely used technique to assess an individual's ability to detoxify is the measurement of the pharmacokinetics of a model compound. Broadly speaking, model substrates may be divided into either drugs which are used to assess factors which modulate the activity of the drug-metabolizing enzymes, or drugs which are used to determine polymorphism in drug metabolism (90). Obviously, there is no reason why a particular drug could not be used for both purposes.

Several investigators have used the rate of plasma clearance of antipyrine (the antipyrine test) and/or aminopyrine, the most widely used model substrates, as a means of estimating drug-metabolizing activity in humans and to qualitatively determine the effect of environmental factors on this activity (86,93). Several reviews have been written on the conceptions and misconceptions concerning the use of model substrates (93,94). Although the model drug approach is logical for a number of reasons, including the ability to control the levels of the drug, the results have

been such that the tests are not applicable to the assessment of overall ability to handle exposure to xenobiotics. In order for a drug to be considered as a model substrate it must possess certain criteria, such that, urinary metabolite excretion can reflect enzyme activity (90). No 'ideal' drug which exhibits all the necessary properties has yet been found. The question also arises whether a test drug can be used to predict the metabolism of other drugs administered to the same patients. Since each drug (xenobiotic) is converted to metabolites by a different combination of hepatic drug-metabolizing isozymes, it would seem unlikely that a single drug could serve as a model for all drugs/xenobiotics (93). Also, only a limited number of many oxidative pathways are represented by the metabolism of antipyrine and aminopyrine, and other model substrates (93).

Theoretically, changes in the disposition of an endogenous compound, or an alteration in the activity of an accessible (blood) enzyme, may provide a useful, non-invasive method to assess an individual's ability to deal with xenobiotics (86,90). Substances used for this purpose include plasma gamma-glutamyltransferase, urinary D-glucaric acid, urinary 6-ß-hydroxycortisol, and plasma bilirubin (86,90). The rationale for selecting these substances as markers of enzyme activity was based largely on observations that the concentrations of these substances in blood or urine were significantly altered in patients undergoing anticonvulsant therapy with either phenytoin or barbiturates (90,95). Although there is no doubt that increases in the excretion of these endogenous compounds is often associated with induction of the drug-metabolizing enzymes, it remains to be shown conclusively that such changes *per se* are proof of enzyme induction (90).

Thus, to date, most attention has been focused on the development of methods for monitoring oxidative biotransformations. Less attention has been aimed at the development of methods for the assessment of phase II biotransformations. Where attempts have been made, the approach has been to measure the excretion of conjugates (or metabolites) of a specific compound(s)/drug(s). Acetaminophen

(paracetamol) and oxazepam are drugs which have commonly been used for this purpose (67,96,97). Unfortunately, these drugs are almost exclusively conjugated via glucuronidation (oxazepam) or a combination of glucuronidation/ sulfation conjugation (acetaminophen). Acetaminophen, to a limited extent, also undergoes mercapturic acid formation. No drug exists that forms, simultaneously, conjugates representative of the four conjugation pathways discussed. Therefore, it is not possible to use one drug to determine an individual's ability to detoxify via all the alternative pathways.

Some attempts have been made to determine the ratio of urinary conjugates (glucuronide:sulfate) under conditions in which sulfate or sulfate sources were limited or increased (11,98). Glucuronide and sulfate pathways may be regarded as competing pathways. However, of these two routes of conjugation, sulfation is more likely to be limited. Not only does sulfation require higher energy expenditure, but also, endogenous sulfate is usually more limited than endogenous glucuronic acid. Sulfate conjugation tends to predominate at low xenobiotic levels and with compounds having small, chemically simple structures. With increasing xenobiotic levels or decreasing sulfate levels, the ratio of glucuronidation to sulfation increases. Although alterations in the ratio were noted, again the studies dealt with a specific drug and the applicability to other xenobiotics is questionable.

Murano et al. (99) determined the daily urinary excretion levels of glucuronides and sulfates and compared these conjugate levels in a free-living population of adult male volunteers. The 40 free-living males in this study were found to predominantly excrete conjugates of sulfates as compared to glucuronides (mean value of 7.65 mmol of sulfoconjugate/day compared to 0.848 mmol glucuronic acid conjugate/day). It was noted that with limited sulfation glucuronidation tended to increase, whereas, with limited glucuronidation there was no increase in sulfation.

Thus, most research studies dealing with the development of methods to assess detoxication have dealt with either specific xenobiotics or with the inducibility,

by various factors, of the xenobiotic metabolizing enzyme activities and not with an overall index of assessment based on the activity of the major conjugation pathways.

Additionally, it is generally accepted that the liver is the predominant site of detoxification whether dealing with phase I or II reactions. It is equally recognized that extra-hepatic tissues do metabolize drugs and foreign substances, although the degree of overall involvement of these tissues in detoxication is not as clear. Therefore, the approach of studying whole body detoxification, from the perspective of the conjugation pathways, would essentially circumvent the need to determine the separate involvements of the hepatic and extra-hepatic tissues. Thus, the proposed study will involve examining whole body detoxification from the perspective of the conjugation pathways rather than specific xenobiotics.

That the detoxication pathways are affected by a host of factors ranging from exposure to environmental chemicals (including drugs), to different diets, and to diseases is not surprising. Further complications include the nonspecificity of the responsible enzyme systems, xenobiotic interaction and competition for enzymes, the inducibility of the enzyme systems (i.e., cruciferous vegetables), and competition for the requisite endogenous molecules. It is equally not surprising then that attempts to measure individual abilities to detoxify xenobiotics are very limited, while at the same time there exists an obvious need for assessment methods.

It is evident that a need exists for establishing the pattern of conjugate urinary excretion on a population basis. It is essential to determine the range of excretion of urinary conjugates in a normal population and to determine the overall effect of a number of critical host factors on this range. Once this has been accomplished, a powerful predictive tool will have been developed which can then be used to foretell an individual's reaction to drugs, level of exposure to chemicals in his/her environment, ability to cope with such exposure, etc. The model that has been described in this proposal offers a way to develop the range of excretion of urinary conjugates in a normal population, and to assess the effect of confounding factors on this range.

This model would also permit a comparison of the levels of urinary excretion for the different conjugation pathways and help to determine interaction amongst the pathways.

Hence, the purposes of this research are to quantitatively assess the urinary excretion of the four conjugation pathways discussed in order to establish normal urinary excretion ranges for these conjugates and to develop a profile of detoxification.

The primary objectives of this research are:

1) To determine the usual urinary level of conjugates of four different pathways and the sum of these pathways;
2) To determine the endogenous level, i.e., without dietary nonnutrients, of the four pathways and their sum by feeding a semisynthetic diet;
3) To determine the time required to attain endogenous levels of the four pathways;
4) To determine if variance is lowered by the introduction of a semisynthetic diet;
5) To index individuals into high and low detoxification profile groups.

It is assumed that, when taken as a whole, the enzymatic detoxification system, is (a) relatively nonspecific, (b) the biliary excretion of conjugates should not significantly alter the urinary profile or pattern, (c) the urinary conjugates examined are essentially completely ionized at urinary pH, and (d) the urinary excretory products are predominantly conjugates of the four pathways to be analyzed. To minimize the influence of diet and to determine endogenous levels of conjugation, a semisynthetic diet was given to carefully selected subjects. It was assumed that a urinary profile, generated in the presence of minimal dietary nonnutrient compounds, would be indicative of the conjugation of endogenous compounds. The profile would represent a baseline, or reference point, of the body's ability to detoxify endogenous compounds, thereby reflecting the contribution of environmental factors.

METHODOLOGY

Design

The urinary excretion of the end products of four detoxication pathways, i.e., sulfate, glucuronide, amino acid conjugates, and mercapturates, was monitored in 18 adult males over a 3-day period to establish the typical levels of excretion for each subject. A multivariate analysis of the four pathways was used to reflect an individual's overall index of detoxification.

Having established the individual's typical levels of each pathway, the contribution of nonnutrient compounds to each pathway was determined by placing the individual on a semisynthetic diet and monitoring urinary conjugates for seven days. The differences between typical and baseline levels, established while on the semisynthetic diet, are assumed to be due primarily to dietary nonnutrient compounds.

The use of each individual as his own control and the subject selection criteria employed were designed to minimize genetic and most extraneous environmental sources of variation in detoxification.

Selection of Subjects

The total of 18 participants was selected from among volunteers associated with Virginia Tech. The subjects were recruited through advertisements in the campus paper and via distribution of posters and fliers around campus and the town of Blacksburg. The advertisement for the study continued until the desired number of subjects was recruited.

The subjects selected were students between the ages of 22 and 40 years and in good health. Selection criteria (see attached Selection Criteria Survey Questionnaire in **Appendix A**) included being within 10% of desirable weight for height according to the Metropolitan Life Insurance Company height-weight data (revised,

1983), no use of tobacco, no use of drugs or medications, and only a moderate consumption of alcoholic beverages (less than 1 drink per day). Volunteers who were selected and completed the study were compensated.

Several meetings were scheduled for the purpose of explaining the objectives of the study to all persons interested. In addition, the rights and responsibilities of the subject, the possible risks, benefits of participation, and the detailed procedures of data collection were carefully explained. At the conclusion of each meeting, all volunteers who had decided to participate were asked to sign the Informed Consent Form which can be found in **Appendix B**. Personal information was also obtained for contacting purposes, such as the scheduling of the data collection period.

Subjects who were involved in a weight loss or weight gain program were excluded from participation in the study.

Data Collection

During the collection period, subjects were restricted from taking any alcoholic beverages, medications, and consumed only the semisynthetic diet, except for the three days when they were on their self-selected diet. It was emphasized that the subject must report any deviation from restrictions placed on him. This emphasis was an attempt to encourage honesty and cooperation, since the data from subjects who digress from the stipulations of the study would have been excluded.

1. Pre-experimental survey. Each subject was required to complete the "Toxicological Risk Assessment Study Selection Criteria Survey" which can be found in **Appendix A**. This pre-experimental survey was used to select subjects who were within 10% of their desirable weight for height, did not use tobacco or alcohol, social drugs, etc. The survey also attempted to quantify the frequency of exposure to environmental xenobiotics.

To ensure anonymity, each subject was assigned a code number for use on all data that was collected. This assisted in enhancing the honesty and reliability of

answers to delicate questions in the Survey, such as the use of social drugs.

2. Urine collection. Each selected subject was required to collect his urine daily for three consecutive days prior to initiating a change in dietary intake. Following a change in the subject's diet, i.e., introduction of the semisynthetic diet, he was required to collect urine daily for seven days (see Design).

Every subject was given urine collection bottles (Polypropylene, 1 liter) that had been acid-washed and autoclaved. The bottles were clearly labelled with subjects code number and collection date. The handout, "Instructions for Collection and Handling of Urine" (see **Appendix C**) was distributed to each subject and each subject was given an oral explanation of the procedures. The subject returned, on the following day, the previous day's 24-h urine collection, at which time he was given new collection bottles. Additional collection bottles were readily available for subjects requiring more than the original number given to them for a 24-h period. A 24-h period is defined as beginning with the collection of the second voiding on day one, through the first voiding on day two.

Upon the delivery of each 24-h collection, the weight of the subject was obtained and recorded, to determine if weight loss had occurred due to the semisynthetic diet. Questions were also asked concerning any deviations from the collection and handling procedures, specifically, incomplete collections or spillages, and consumption of medications, etc.

The total volume of each specimen was measured and recorded. After thorough mixing, the pH (5.7-7.0, on average, for the entire experimental period) was determined and recorded. Several aliquots were then taken and immediately frozen for future analysis.

3. Dietary intake

Subjects were required to complete a "Daily Food Intake Record" (see **Appendix D**) to determine each subject's diet history, during the initial three days of the study. Therefore, each subject was asked to maintain his usual dietary intake and

to accurately weigh or measure all foods and beverages consumed. The mean daily caloric value of this 3-day intake was used as a basis for establishing the amount of semisynthetic diet to be provided. The semisynthetic diet selected for use was a defined formula with fiber (Enrich, Ross Laboratories).

4. Conjugate Analyses

The assays for the measurement of urinary sulfoconjugates, glucuronides, and glutamine and glycine conjugates and mercapturates have been previously conducted in our laboratories. Sulfoconjugates (**Appendix E**) were determined by the method of Lloyd et al. (100). This method involves the measurement of barium chloride-precipitated sulfate with the atomic absorption spectrophotometer. Glucuronide (**Appendix F**) determination involves a modified naphthoresorcinol method (101). Conjugates of the amino acids glutamine, glycine, and taurine (**Appendix G**) were quantified using a modified method of Bidlingmeyer et al. (102). This method involves derivatization of amino acids with phenylisothiocynate and subsequent separation of derivatives by high performance liquid chromatography. Urinary mercapturates (**Appendix H**) were determined by modification of the method of Seutter-Berlage (103). All analyses were conducted in triplicate.

5. Data Analysis

All the raw data were entered into a Quattro spreadsheet (Borland International) and set up to carry out the necessary computations and transformations. SAS/STAT was used for the statistical analysis (104). Three excretory pathways (amino acid conjugates were not included due to the small sample size that resulted after pooling samples) were analyzed separately using a repeated measure analysis of variance with Helmert transformation to determine the response of the excretion levels of urinary conjugates, over time, to the liquid diet. A multivariate analysis of variance (MNOVA) was used to compare the means of the self-selected diet and the semisynthetic diet for all four pathways.

A principal component analysis (105) and Mahalanobis distance were done on three of the conjugate excretion levels (glucuronides, sulfoconjugates, mercapturates) to determine interactions amongst these pathways. The first principal component and Mahalanobis distance were used as an overall index of detoxification. Amino acid conjugates were not used in this statistical analysis because of the small sample size that resulted due to the necessity of pooling of urine samples.

JOURNAL ARTICLES

The effect of the introduction of a semisynthetic diet on the profile of urinary conjugates excretion[1-2]

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Abstract

The effect of a self-selected and semisynthetic diet on urinary conjugate levels was determined in 18 male adults (22-40 y). Subjects consumed a self-selected diet for three days and a semisynthetic diet for seven days. Glucuronides and mercapturates were quantified using spectrophotometric methods using naphthoresorcinol and Ellman's reagents, respectively. Atomic absorption was used to measure barium chloride-precipitated sulfate for sulfoconjugates. Conjugated amino acids were determined by HPLC of phenylisothiocyanate derivatives. A MANOVA was done to compare the means of the dietary periods and Helmert transformation was done to determine response to dietary change. Mercapturates and amino acid conjugates were most sensitive to dietary change, quantities excreted reduced by about 50% during the semisynthetic diet period (0.27 ± 0.11 vs 0.14 ± 0.02 mmol/24-h; 5.99 vs 3.03 mmol/24-h, respectively). Glucuronides were the least responsive to dietary change with no significant difference between the means of the two diet periods (self-selected diet 2.93 ± 0.77; semisynthetic 3.21 ± 0.29 mmol/24-h). Sulfoconjugate levels were the highest of all the conjugates measured. Sulfoconjugates were initially decreased on the semisynthetic diet (5.28 ± 2.54 mmol/24-h vs. 3.98 mmol/24-h), but by day four, sulfoconjugate excretion began to increase. The increase may have been due to the presence of vanilla flavoring in the liquid diet. In summary, the quantity of conjugates excreted were found to be sensitive to dietary changes, with some pathways more responsive than others.

Introduction

One of the factors which most influences metabolism and the disposition of exogenous chemicals is nutrition (1-5). All the reactions of xenobiotic metabolism are dependent on the nutrient supply of the body (6-7), plus, mammals are constantly

exposed to naturally occurring and synthetic chemicals, that are toxic or carcinogenic, via ingestion of foodstuffs (1). Because diet for man is so varied, it is a most complex variable in ascertaining its role in the rates of toxication or detoxication of drugs and xenobiotics.

In normal human subjects, several dietary manipulations have been identified that can alter the disposition of such model drugs as antipyrine, phenacetin, and theophylline (8-10). Nutritional factors investigated include: 1) varying the proportions of protein, carbohydrate and fat in a daily diet of 2,500 kcal; 2) charcoal broiling of beef; and 3) a high intake of cruciferous vegetables. These single factors can interact with each other in the normal diet and thereby contribute to the well recognized large interindividual variations that occur in drug/xenobiotic metabolism and disposition.

Because of the relationship between toxicology and nutrition, Truhaut and Ferrando (11) recommended that when dealing with toxicological experiments, it is advisable to account for nutritional causes capable of altering the body reactions to toxic substances. Thus, Abbott et al. (12) studied the effect of a purified diet vs. a commercial crude diet on the components of the hepatic mono-oxygenase system of rats. Little or no consistent diet-related difference was observed, nor were results significantly less variable with one or the other diet. These results differ from studies dealing with humans and the effect of diet on xenobiotic metabolism. The discrepancy may be due to the fact that the human diet is so much more varied than

that of laboratory animals. Furthermore, human food is usually cooked which can produce products, possible potent mutagens, that are unlikely to be in the diets of laboratory animals (2). Therefore, a greater difference in effect on drug metabolism would be expected in humans when a varied, self-selected diet is consumed as compared to consumption of a semisynthetic diet.

Most research dealing with the effect of diet on drug (xenobiotic) metabolism has focused on phase I reactions. Few researchers have looked at the effect of diet on the conjugation (phase II) reactions. Therefore, the purpose of this study was to determine the influence of food on conjugate excretion by measuring conjugate levels when individuals consume a semisynthetic diet that minimizes exposure to xenobiotics, and to determine the time required for conjugate levels to respond to the diet modification.

Methods

Subjects

The subjects were 18 male Caucasian students at Virginia Polytechnic Institute and State University which is situated in a rural mountainous area. Subjects were between the ages of 22-40 yr and selected from a number of respondents to a questionnaire which contained questions concerning health, smoking and drinking habits, and willingness to abstain from alcohol, tobacco, and drug use. Subjects performed all their normal daily activities during their participation in the study, except that no drug or medication was allowed and that, from the fourth day until

completion of the study, a semisynthetic/liquid diet was consumed as a total replacement for normal dietary foodstuffs. Feeding was on a demand basis without restriction as to time.

Diets

During the first three days, subjects consumed their usual self-selected diets and kept records of their food consumption for the purpose of determing their average caloric intake. For the next seven days subjects were given a commercially available, chemically defined liquid diet, Enrich with Fiber (Ross Labs.). The semisynthetic diet (**Table 1**) was selected for its acceptability to humans over a period of several days, its capacity to be used as a total feeding regimen, and its theoretical lack of possible xenobiotics except for vanilla which was used as a flavoring agent. Subjects were given as many 8 oz cans as were necessary to maintain the caloric intake as calculated from the three day self-selected diet. Body weights were measured daily to ascertain that sufficient semisynthetic diet was being consumed by each subject. During the seven days when subjects were consuming the semisynthetic diet, no other foods or drugs were allowed. No other restrictions were put on the subjects' lifestyles or consumption of water.

Sample collection and analysis

Urine was collected for each day (a total of 10) in 1 L plastic bottles that had been thoroughly cleaned and autoclaved. Total daily urine volumes were measured and recorded. After mixing the 24-h urine collection, aliquots were taken for the

four conjugation pathways (glucuronide, sulfate, mercapturates, and amino acid conjugates). All samples were frozen the same day of collection and kept at -20^0 C until analysis.

The modified naphthoresorcinol (NR) method of Mazzuchin et al. (13) was used for the quantification of urinary conjugated glucuronic acid. Interference by glucose was selectively eliminated with glucose oxidase and free glucuronic acid was decomposed to a NR-insensitive product using sodium hydroxide. The methodology permits the determination of conjugated glucuronic acid even though the urine may contain high glucose concentrations and free glucuronic acid. The color developed was extracted with ethyl acetate and the absorbance was measured, against a blank, at 564 nm in a Spectronic 601 (Milton Roy). Concentrations of conjugated glucuronic acid were determined from a standard curve which had been developed using phenolphthalein glucuronide.

Quantification of sulfoconjugates was determined by using a modified method of Lloyd et al. (14) and was calculated as the difference between inorganic sulfate before and after hydrolysis with fuming nitric acid. Inorganic sulfate was precipitated as a barium sulfate pellet, washed three times, dissolved in ethylene diaminetetraacetate solution (EDTA) and measured with a spectrophotometer (Perkin-Elmer 2100). Varying concentrations of aqueous sodium sulfate, which had been precipitated with barium chloride and washed three times, similar to the unknown urine samples, were used to determine the standard curve.

A modified colormetric method of Seutter-Berlage et al. (15) was used to determine mercapturic acids. The yellow color that developed as a result of using Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) was measured at 412 nm with a Spectronic 601 (Milton Roy). Mercapturic acid concentration was determined by the difference between free sulfhydryls (after reduction with $NaBH_4$) and sulfhydryls after hydrolysis with 5N NaOH. Standard curves for prehydrolysis and posthydrolysis were developed separately, using N-acetylcysteine as the standard.

Urinary conjugated glycine, glutamine, and taurine were measured using the method of Bidlingmeyer et al. (16). This method is based on the derivatization of amino acids with phenylisothiocyanate (Edman's reagent) to phenylthiocarbamyl derivatives, which can then be separated by high performance liquid chromatography. The PICO.TAG chromatography system (Water Assoc.) was used with a fixed wavelength detector (254 nm) and a solvent system consisting of eluent A (buffer) and eluent B (acetonitrile, methanol, water). The quantification of the conjugated compounds was determined by calculating the differences of amino acid before and after HCl hydrolysis. Due to the high cost of analysis, amino acid conjugate determinations were done on pooled samples consisting of three randomly formed groups, each containing six subjects. Only pooled urines from the third day of the self-selected diet and the second, fifth, and seventh day of the semisynthetic diet were analyzed.

Statistical Analysis

Repeated measures analysis of variance, with Helmert transformation, was used to determine the response of the excretion levels of the urinary conjugates to the semisynthetic diet over time. To compare the means of the self-selected diet and the semisynthetic diet, a multivariate analysis of variance was used (17).

Results

Compliance with the use of the semisynthetic diet and other restrictions was excellent for all subjects. Body weight changes during the semisynthetic diet period were limited to a mean change of 0.65 kg. Although 13 subjects lost weight, just one subject lost as much as 2 kg.

The quantity of urinary conjugates excreted for both the self-selected diet and semisynthetic diet are given in **Table 2.** For the self-selected dietary period, total amino acid conjugates, which represent the total of glycine (4.75 mmol/24-h), glutamine (1.18 mmol/24-h), and taurine (0.07 mmol/24-h) conjugates, were excreted the most, with a value of 5.99 mmol/24-h on the third day of the self-selected diet. The quantity of sulfoconjugates excreted was the second highest with a range of 5.10 to 5.48 mmol/24-h on the self-selected diet. Yet, if sulfoconjugates quantities are compared with the quantities of individual amino acid conjugate excreted, then the former had the highest excreted amount. The third highest were the glucronides (2.87 to 2.98 mmol/24-h), with the mercapturates (0.26 to 0.29 mmol/24-h) having the lowest excretion.

During the entire semisynthetic diet period the quantities of sulfoconjugate measured (3.98 to 8.52 mmol/24-h) were the highest of all four pathways measured. Glucuronide quantities (3.38 mmol/24-h) were the second highest on the first day of the semisynthetic diet, but were comparable to total amino acid conjugates (3.06 mmol/24-h) by the seventh day of the semisynthetic diet. Although amino acid conjugates for the first day of the semisynthetic diet were not analyzed, the quantities of excretion measured for days two and five of the semisynthetic diet indicated that the amino acid conjugates were the second highest when expressed as total amino acid conjugates. A decrease occurred by the fifth day (5.30 mmol/24-h) and by the seventh day of the semisynthetic diet, excretion levels dropped precipitously to 3.03 mmol/24-h. As in the self-selected dietary period, mercapturate excretion (0.12 to 0.16 mmol/24-h) was the lowest of all the conjugate excretion levels measured.

The mean of three days of a self-selected diet, and the mean of seven days of a semisynthetic diet for three of the conjugation pathways measured are presented in **Table 3**. The mean excretion level (0.14 mmol/24-h) of mercapturates during the seven days of the semisynthetic diet was significantly lower ($p<0.0001$, paired Student's t-test) than the mean (0.27 mmol/24-h) of the three days of the self-selected diet. The mean excretion levels of the self-selected diet for the glucuronides and sulfoconjugates were not found to be significantly different from the mean excretion levels of these conjugates while subjects consumed the semisynthetic diet.

The excretion levels of the three amino acids measured are represented in

Figure 1. Glycine is the most predominant amino acid contributing to more than 70% of the total amino acid conjugate excretion, while taurine excretion contributed to less than 5% of the total excretion. Taurine excretion initially increased (more than doubled) while subjects were consuming the semisynthetic diet and later decreased to amounts comparable to the self-selected diet period. Both glycine and glutamine excretion were reduced during the semisynthetic dietary period, although there seemed to be a greater reduction in the glycine excretion levels.

Discussion

Mean excretion levels for all four types of conjugates measured, while on the self-selected diet, were similar to levels previously measured in our laboratory and by other researchers, except for the glucuronides (13,18,19,20). The values reported by Murano et al. (18) were only 29% of the recorded mean values of the present study for a similar 3-day diet period. A mathematical correction of the former reported values resulted in excretion amounts that conform more with those of the present study (2.93 mmol/24-h) and with values reported by other researchers, i.e., Muzzuchin et al. (13)(maximum 2.52 mmol/24-h), and Fishman and Green (20) (maximum 2.38 mmol/24-h).

Although most of the conjugate types studied responded to dietary change (**Table 2**), mercapturates seemed the most responsive to the introduction of the semisynthetic diet. Mercapturates were decreased by about 50%, a decrease that persisted for the duration of the dietary period. The three days of the self-selected

diet were found to be significantly different ($p<0.004$) from the mean of the subsequent seven days of the semisynthetic diet, although there was no significant difference within the seven days of the semisynthetic dietary period, using Helmert transformation for repeated measures analysis. This significant difference was also found using the Student's t-test ($p<0.0001$) (**Table 3.**). The lack of significance within the seven days of the semisynthetic dietary period, implied that once excretion levels were decreased they stabilized and remained fairly constant.

Because mercapturates were the most responsive to dietary change, formation of these conjugates would appear to involve mostly exogenous compounds rather than endogenous compounds. Thus, mercapturates appeared to be a better indicator of dietary change than the other conjugates studied. The diet can be a potential source of electrophilic substances which may have been produced during processing or as a natural product of the food. Once a major source of these foreign compounds was removed (the self-selected diet), mercapturate excretion decreased and remained low. Van Doorn et al. (21) found that when exposure of one or more electrophilic substances had occurred, then urinary mercapturate excretion increased. The reverse, also, would seem to be true - when exposure to electrophilic substances is reduced, urinary mercapturates should decrease.

Amino acid conjugate excretion were also very responsive to dietary change. Although these conjugates did not respond as quickly as the mercapturates to the dietary change, a decrease was not seen until day five of the semisynthetic diet,

excretion levels were also reduced by about 50% by the end of the semisynthetic dietary period. Amino acid conjugate excretion on day seven of the semisynthetic diet was found to be significantly different ($p<0.01$) from the excretion levels of these conjugates on the third day of the self-selected diet, using the Student's t-test (**Table 2**). The drop in the total amino acid conjugate excretion was best represented by glycine excretion levels, although glutamine excretion appeared to be decreasing as well.

Sulfoconjugate levels on all three days of the self-selected diet were found not to be statistically different from the mean of the seven days of the semisynthetic diet, using Helmert transformation for repeated measures analysis (**Table 2**). Although there was no significant difference among the excretion levels of the three days of the self-selected dietary period, sulfoconjugate excretion for each day of the semisynthetic diet was significantly different ($p<0.05$) from subsequent days, within the semisynthetic dietary period. Although there was no statistical difference between the two dietary periods, which was also substantiated by the Student's t-test (**Table 3.**), the significant difference in excretion levels that were observed during the semisynthetic dietary period would indicate that there were dynamic changes, i.e., bodily adaptations to the new diet, etc., that occurred during the dietary period.

After day three of the semisynthetic diet, sulfoconjugate excretion increased and the increase continued until the end of the dietary period (**Table 2**). The increase in sulfoconjugate excretion may have been due, in part, to certain substances

that were in the semisynthetic diet that were preferentially conjugated via sulfation. The semisynthetic diet product used was flavored with vanilla, which is a 3-ethoxy-4-hydroxybenzaldehyde. Mamer et al. (22) found metabolites of this compound in patients who had been given synthetic diets flavored with vanilla. Because of its structure, the benzaldehyde is more likely to be conjugated via sulfation or glucuronidation rather than with glutathione. This would explain the lack of increase in mercapturate excretion while on the semisynthetic diet. A sulfoconjugate increase rather than glucuronide increase may have been due to the favorable competition of sulfation with glucuronidation because of the low K_m value of the sulfotransferases. Why there was a lagtime of three days before sulfoconjugates began to increase is not known.

Glucuronide excretion levels seemed to be the least affected by the change in diet. Although there was a slight increase in the excretion of these conjugates, the increase was not significant. This insensitivity to diet change may have been due to the fact that glucuronidation is, possibly, utilized more for the metabolism of endogenous compounds rather than exogenous compounds. Glucuronide excretion levels may also have been masked by the high excretion of the sulfoconjugates. Sulfoconjugation and glucuronidation are competing pathways for many of the same xenobiotics. Because of the low K_m of the sulfotransferases, the enzymes that catalyze the sulfoconjugation of xenobiotics, sulfoconjugation is usually the more predominant of the two pathways. Glucuronidation becomes the dominant

conjugation reaction when xenobiotic levels go up or when sulfate is limiting in the diet (23,24). On the semisynthetic diet, xenobiotic concentration was low and there was sufficient sulfate in the diet. The possibility also exists that the municipal water supplied to the subjects for drinking purposes may have been an additional, extraneous, source of sulfate. Hindmarsh et al. (25) found that increased sulfate levels in drinking water increased the sulfate concentration in serum, further suggesting that sulfate was not limiting in the present study.

In summary, this study demonstrated that conjugate excretion levels are sensitive to dietary changes. Some of the conjugate pathways are more responsive to this dietary change than others. Also, when a response did occur, it was generally very quick, with changes appearing as soon as the first day of dietary change.

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TABLE 1. Nutrient breakdown of the semisynthetic diet Enrich* with fiber (Ross Labs)

NUTRIENTS	% CALORIES**	GRAMS/8 OZ. CAN
Protein	14.5	9.4
Sodium and calcium caseinates		1.9
Soy protein		0.3
Fat (corn oil)	30.5	8.8***
Polyunsaturated fatty acids		4.8
Saturated fatty acids		1.3
Carbohydrates	55.0	38.3
Hydrolyzed cornstarch		22.2
Sucrose		12.2
Soy polysaccharide		3.8

*About six 8-oz. cans provides 100% of U.S.RDA for vitamin and minerals and contains about 23 g dietary fiber.

**8 oz. can contains 260 calories.

***Fatty acids equal approximately 95% of total fat.

TABLE 2. Urinary excretion levels of 18 subjects on three days of a self-selected diet and seven days on a semisynthetic diet.

DIET	CONJUGATES (mmol/24 h)				
Day	Glucuronides	Sulfoconjugates	Mercapturates	Amino Acid Conjugates†	Total Conjugates
Self-Selected					
1	2.93±0.94*	5.48±3.86	0.26±0.07[b]	--**	
2	2.87±1.03	5.10±2.92	0.28±0.15[b]	--	
3	2.98±0.96	5.28±4.09	0.29±0.19[b]	5.99±0.45[a]	14.54
Semisynthetic					
1	3.38±1.27	4.52±1.97[b]	0.15±0.07	--	
2	3.18±0.94	4.18±1.92[b]	0.16±0.08	6.24±0.87	13.76
3	3.21±0.80	3.98±1.73[b]	0.13±0.05	--	
4	3.73±1.20	5.20±1.57[b]	0.13±0.06	--	
5	2.85±0.67	5.53±2.11[b]	0.12±0.04	5.30±0.69	13.80
6	3.04±0.72	6.16±2.94[b]	0.15±0.06	--	
7	3.05±0.94	8.52±3.59	0.16±0.05	3.06±0.18	14.79

*Mean ± SD.
†Only 4 days determined on pooled samples (three groups of six subjects each) and represents the total of glycine, glutamine, and taurine conjugate excretion.
**Not analyzed.
[a]Significant at $P \leq 0.01$ using Student's t-test compared to semisynthetic diet day seven.
[b]Each day is significantly different ($P \leq 0.05$) from the mean of the succeeding days as determined by Helmert transformation of repeated measures analysis.

TABLE 3. Comparison of mean urinary excretion of conjugates for 18 subjects who consumed a self-selected diet for three days and then a semisynthetic diet for seven days.

DIET	CONJUGATES (mmol/24 h)		
	Glucuronides	Sulfoconjugates	Mercapturates
Self-selected (3 d)	2.93±0.77*	5.28±2.54	0.27±0.11[a]
Semisynthetic (7 d)	3.21±0.29	5.44±1.56	0.14±0.02

*Mean ± SD
[a]Significant at P≤0.0001.

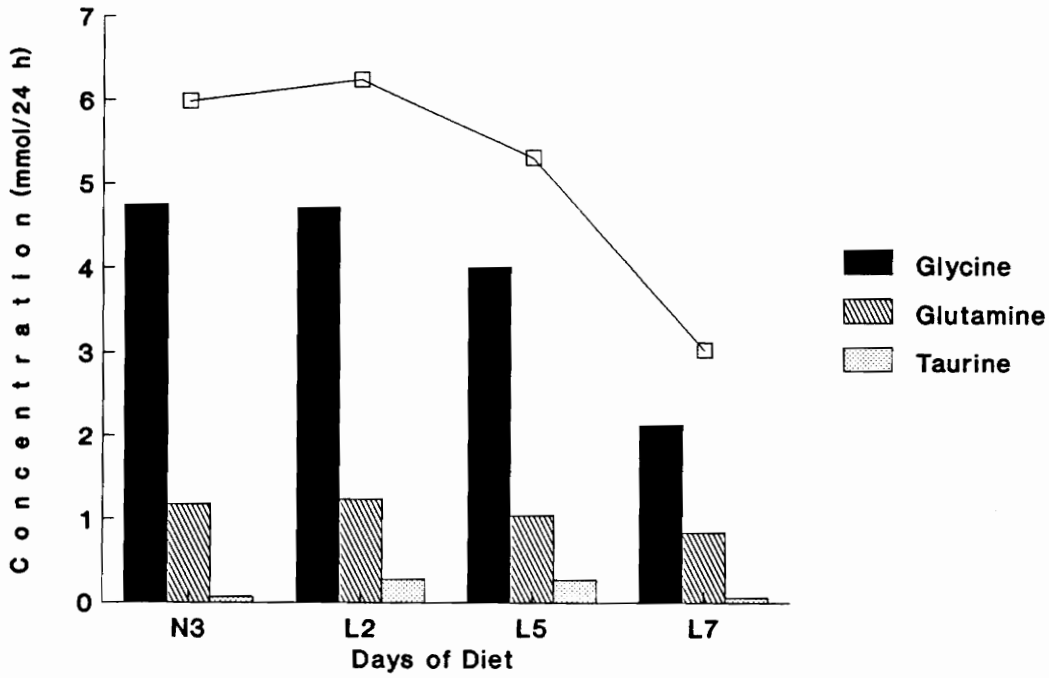

FIG 1. The excretion of glycine, glutamine, and taurine conjugates of 18 subjects consuming a self-selected diet for three days and a semisynthetic diet for seven days. (N3 = third day of self-selected diet; L2, L5, L7 = second, fifth, seventh day of semisynthetic diet.)

A multivariate approach to the development of a urinary conjugate profile for adult males on a semisynthetic diet

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KEY WORDS Multivariate analysis, urinary conjugates, detoxification profile, semisynthetic diet, glucuronides, sulfoconjugates, mercapturates, amino acid conjugates

Abstract

Urinary conjugates were quantified in 18 male adults (22-40 y) to establish normal ranges for these conjugates and to develop a profile of detoxification using a multivariate approach. Subjects consumed for three days a self-selected diet, and seven days a semisynthetic diet. The glucuronide and mercapturate conjugates were quantified using a spectrophotometric method with naphthoresorcinol and Ellman's reagent, respectively. Atomic absorption was used to measure barium chloride-precipitated sulfate for sulfoconjugates. Conjugated amino acids were determined from phenylisothiocyanate derivatives by HPLC. Four methods for developing 'normal' ranges were presented: mean ± SD, percentiles, principal component analysis (princomp), Mahalanobis distance (distquan). The four methods were compared. The princomp and distquan methods were stressed because they are a multivariate approach which integrates the values for all the conjugate pathways and their interaction in order to develop a single value. This single value would then be representative of an individual's total, or overall, detoxification index relative to others in the group studied.

Introduction

The detoxification of compounds foreign to the intermediary metabolism of mammals is a process that involves a series of metabolic conversions catalyzed by enzymes, a number of nonspecific reactions, and final excretion (1-3). The commonly accepted view is that the metabolic pathways encountered in detoxification represent a series of steps by which foreign compounds are "prepared" for excretion via phase I and phase II reactions. Phase I reactions both enhance the water solubility of the xenobiotics and, also, generate a substrate for phase II metabolism (4-5). Phase II reactions - also known as conjugation reactions - involve the combination of an endogenous conjugating agent with a foreign compound, or metabolite of phase I, leading to the formation of a water-soluble product which can be readily excreted. The conjugation reactions are generally regarded as true 'detoxi-

fication' reactions and changes in the ability of the body to conjugate xenobiotics leads to large changes in toxicity of the xenobiotic (4,6). Thus, it would seem appropriate to determine the detoxification profile of an individual by measuring urinary conjugate levels.

Most research studies dealing with the development of methods to assess detoxification have dealt with either specific xenobiotics or with the inducibility, by various factors, of the xenobiotic metabolizing enzyme activities and not with an overall index of assessment based on the activity of the major conjugation pathways (7-10). Several investigators have used the rate of plasma clearance of antipyrine (the antipyrine test) and/or aminopyrine, as a means of estimating drug-metabolizing activity in humans (7,9). Although the model drug approach is logical for a number of reasons, the question arises whether a test drug can be used to predict the metabolism of other drugs administered to the same patient. Since each drug (xenobiotic) is converted to metabolites by a different combination of hepatic drug-metabolizing isozymes, it would seem unlikely that a single drug could serve as a model for all drugs/xenobiotics.

Additionally, it is generally accepted that the liver is the predominant site of detoxification whether dealing with phase I or II reactions. It is equally recognized that extrahepatic tissues do metabolize drugs and foreign substances, although the degree of overall involvement of these tissues in detoxification is not as clear (11-12). Therefore, the approach of studying whole body detoxification, from the perspective of the conjugation pathways, would essentially circumvent the need to determine the separate involvements of the hepatic and extrahepatic tissues. Therefore, the propose of the present study was to examine whole body detoxification from the perspective of the conjugation pathways rather than specific xenobiotics, and to quantitatively assess the excretion levels of the four conjugation pathways in order to establish normal ranges for excretion of these conjugates and to develop a profile of detoxification.

Methods

Subjects

The subjects were 18 male Caucasian students at Virginia Polytechnic Institute and State University which is situated in a rural mountainous area. Subjects were between the ages of 22-40 y and selected from a number of respondants to a questionnaire which contained questions concerning health, smoking and drinking habits, and willingness to abstain from alcohol, tobacco, and drug use. Subjects performed all their normal daily activities during their participation in the study, except that no drug or medication was allowed, and from the fourth day until completion of the study, a semisynthetic/liquid diet was consumed as a total replacement for normal dietary foodstuffs. Consumption was on a demand basis without restriction as to time of feeding. Informed consent was obtained from all subjects and the study was approved by the Human Subjects Review Board for human research at Virginia Polytechnic Institute and State University.

Diets

During the first three days, subjects consumed their usual diets (self-selected diets) and kept records of their food consumption for the purpose of determing their average caloric intake. For the next seven days subjects were given a commercially available chemically defined liquid diet, Enrich with Fiber (Ross Labs.). The semisynthetic diet was selected for its acceptability to humans over a period of several days, its capacity to be used as a total feeding regimen, and its theoretical lack of possible xenobiotics except for vanilla which was used as a flavoring agent. Subjects were given as many 8-oz cans as were necessary to maintain their usual caloric intake as calculated from the 3-day self-selected diet. Body weights were measured daily to ascertain that sufficient semisynthetic diet was being given to each subject. During the seven days when subjects were consuming the semisynthetic diet, no other foods or drugs were allowed. No other restrictions were placed on the subjects lifestyles or consumption of water.

Sample collection and analysis

Urine was collected for each day (a total of 10) in 1 L plastic bottles that had been throughly cleaned and autoclaved. Total daily urine volumes were measured and recorded. After mixing the 24-h urine collection, aliquots were taken for the four conjugation pathways (glucuronidation, sulfation, mercapturate formation, and amino acid). All samples were frozen the same day of collection and kept at -20^0 C until analysis.

The modified naphthoresorcinol (NR) method of Mazzuchin et al. (13) was used for the quantification of urinary conjugated glucuronic acid. Interference by glucose was selectively eliminated with glucose oxidase, and free glucuronic acid was decomposed to a NR-insensitive product using sodium hydroxide. This methodology permits the determination of conjugated glucuronic acid even though the urine may contain high glucose concentrations and free glucuronic acid. The color developed was extracted with ethyl acetate and the absorbance was measured, against a blank, at 564 nm in a Spectronic 601 (Milton Roy). Concentrations of conjugated glucuronic acid were determined from a standard curve which had been developed using phenolphthalein glucuronide.

Quantification of sulfoconjugates was determined by using a modified method of Lloyd et al. (14) and was calculated as the difference of inorganic sulfate before and after hydrolysis with fuming nitric acid. Inorganic sulfate was percipitated as a barium sulfate pellet, washed three times, dissolved in ethylene diaminetetera-acetate solution, and read on the Atomic Absorption Spectrophotometer 2100 (Perkin-Elmer). Varying concentrations of aqueous sodium sulfate, which had been precipitated with barium chloride and washed three times, similar to the unknown urine samples, were used to determine the standard curve.

A modified colormetric method of Seutter-Berlage et al. (15) was used to determine mercapturic acids. The yellow color that is developed as a result of using Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid was measured at 412 nm with a

Spectronic 601 (Milton Roy). Mercapturic acids concentration was determined by difference of free sulfhydryls (after reduction with $NaBH_4$) and sulfhydryls after hydrolysis with 5N NaOH. Standard curves for prehydrolysis and posthydrolysis were developed separately, using N-acetylcysteine as the standard.

Urinary conjugated glycine, glutamine, and taurine were measured using the method of Bidlingmeyer et al. (16). This method is based on the derivatization of amino acids with phenylisothiocyanate (Edman's reagent to the phenylthiocarbamyl derivatives which can then be separated by high performance liquid chromotography. The PICO.TAG chromatography system (Water Assoc.) was used with a fixed wavelength detector (254 nm) and a solvent system consisting of eluent A (buffer) and eluent B (acetonitrile, methanol, water). The quantification of the conjugated compounds was done by calculating the differences of amino acid before and after HCl hydrolysis. Amino acid conjugate determinations were done on pooled samples. Samples were pooled in to three groups, each containing six subjects. Analysis was done only on the third day of the self-selected diet and the second, fifth and seventh day of the semisynthetic diet. This was necessitated by the high cost of the analytical proceedure.

Statistical Analysis

The data (self-selected diet day three, semisynthetic diet day two) were analyzed to determine the mean ± SD, and the sixteenth and eighty-fourth percentiles. Principal component analysis (17) and Mahalanobis distance (18) were also performed to determine the overall, or total, detoxification profile of an individual.

Results

'Reference values' were developed for the four urinary conjugates measured using four different statistical procedures, i.e., mean ± 1 SD; nonparametric percentile estimates; principal component analysis; and Mahalanobis distance. A comparison of the mean ± 1 SD with nonparametric percentile estimates for the sixteenth and eighty-fourth percentiles, which are equivalent to 68% of the

population or the mean ± 1 SD are shown in **Table 1**. The sixteenth and eight-fourth percentiles were chosen because the small sample size (n = 18) did not allow for calculation of the fifth and ninty-fifth percentile which would have be equivalent to the mean ± 2 SD or 95% of the population. Only the mean ± 1 SD was determined for the amino acid conjugates because the small sample size (n = 3) did not permit the determination of percentiles. Mean values were represented as an upper limit (mean + SD) and a lower limit (mean - SD) in order to facilitate comparisons between the lower and upper limits of the percentile method.

The principal component (princomp) and Mahalanobis distances (distquan) values of three conjugation pathways (glucuronides, sulfoconjugates, and mercapturates) determined for the 18 male subjects are presented in **Table 2**. The princomp and distquan values were determined both for the third day of the self-selected diet and the second day of the semisynthetic diet.

Discussion

Although subjects consumed the semisynthetic diet for a period of seven days, only values for day two of the semisynthetic diet were used to develop 'reference values'. Day two was chosen because of being a more reasonable cut-off period for a clinical setting, i.e., patient should not find it too unreasonable to consume a semisynthetic diet for two days and to collect 24-h urine samples. Also, by this time, conjugates levels have begun to decrease suggesting a sufficient wash-out period from the self-selected diet (see previous paper). The last day (day three) of the self-selected diet was chosen as a point of reference for comparison.

The most common procedure for deriving the reference range for many standard clinical tests used for screening subjects is the arithmetic mean, plus or minus, a multiple of the standard deviation of a series of observations (19). The mean ± 2 SD is taken as the upper and lower limits for 95% of the population. The inference that this range contains within it approximately 95% of the population values is based on three assumptions: a) that the sample size is large enough so that

degrees of freedom may be ignored; b) that the sample is truly random, i.e., the observations have probability laws that are independent and identically distributed; c) that the distribution of the population of observations from which the sample is taken should be a close approximation to the Gaussian (19-22).

Herrera (22) felt that the use of the mean ± 2 SD for determining normal ranges was misleading - especially when the distribution was either skewed, peaked, or flat. If the data are neither Gaussian nor log-Gaussian, and are treated as such, the results obtained can be severely biased. Herrera (22), Reed et al (23), and Mainland (24) preferred the use of nonparametric methods for estimating the normal range because, regardless of the underlying form of the statistical population from which the data are obtained, the methods are applicable.

Both methods are presented for comparison (**Table 1**). For both diet treatments, the upper limit for the percentile method is consistently higher than the upper limit using the mean ± SD method. The percentile method gave a higher upper limit because our data was slightly skewed to the right and percentiles would be more sensitive to a skewed distribution. Also, if the range is computed for each conjugate level for both days, it becomes obvious that the ranges are larger using the percentile method than the mean ± SD, e.g., 1.91 mmol/24 h of glucuronides using the mean ± SD and 2.58 mmol/24 h for the percentile method. Yet, generally the two methods are comparable, and both have the same short-coming in that they are univariate measurements and cannot, therefore, give an overall, or total, detoxification index.

Two methods, based on a multivariate analysis of the data, principal component analysis (17) and the Mahalanobis distance (18), were used to study the multivariate structure of the data. The first principal component and the Mahalanobis distance values are presented in **Table 2**. A principal component analysis helps to describe the covariance structure of a data set by developing a few linear combinations of the original variables (18,25). Algebraically, principal components

are particular linear combinations of the p random variables X_1, X_2,...., X_p. Geometrically, these linear combinations represent the selection of a new coordinate system obtained by rotating the original system with the random variables as the coordinate axes. The new axes represent the directions with maximum variability and provide a simpler and more parsimonious description of the covariance structure. Since the first principal component is the axis along which most of the variation of the data occurs, this component is proposed as an overall measure of detoxification ability.

The distance measure, derived from the distquan method, for each individual, is based an a standarized distance from the mean of the data. If $\mathbf{x}_i$ is the vector mean for the three variables and S is the sample covariance matrix, the usual Mahalanobis distance for a given observation $\mathbf{x}_i$ is given by:

$$\mathbf{D}_i = (\mathbf{x}_i - \mathbf{x})' \mathbf{S}^{-1} (\mathbf{x}_i - \mathbf{x}).$$

However, one problem with this approach is that the observation ,$\mathbf{x}_i$, is included in the calculation of S. To avoid any problems with extreme outliers, the sample mean vector and covariance matrix are substituted with $\mathbf{x}_i$ deleted when computing $\mathbf{D}_i$. This technique of deleting one observation is similar in spirit to jackknifing (26).

If the data are multivariate normally distributed, then the $\mathbf{D}_i$ values are proportional to a random variable distribution with an F distribution with $v_1 = \mathbf{p} = 3$ and $v_2 = \mathbf{n\text{-}1\text{-}p} = 14$ degrees of freedom. The $\mathbf{D}_i$ values can be transformed into percentile scores by inverting this F distribution. Note that, provided the data are not too nonnormal, this score provides some measure of how close the observations are to the mean, as scaled by the covariance. In the univariate case, this was similaar to squaring z-scores.

Thus, the princomp and distquan methods provided two scores of detoxification output based on multivariate considerations. The following paragraphs discuss how these values may be interpreted.

A score close to 0.0 on the princomp method would indicate that the

individual's overall excretion levels, considering all three pathways, was very close to the mean. For example, subject number 27 scored a 0.022 while on the semisynthetic diet, meaning that his excretion profile was average and a doctor could assume that this individual would metabolize medication given to him as anticipated by the drug company. Yet an individual with a score of 1.464, or -1.730 would be farther from the norm and a doctor would then have to be careful, when prescribing medication, to keep the individual under closer observation for possible side effects from the drug. Because the princomp value is similar to a Z-score, the upper and lower limits of normality can be defined as $\pm$ 1.96, which is equivalent to 95% of the population. Therefore, an individual with a score of 2.125, for example, should be observed closely being outside the normal range.

An individual with a distquan score of 0.00 means that he was at the center of the data, and therefore, closer to the mean total excretion levels for the population. A score of 5.276 (subject #7, self-selected diet) would mean that this individual has an excretion level which is very different from the population. Such a value would act as an alert to a clinician who was prescribing medications for this individual. A score of 0.004 (subject #1, semisynthethic diet) suggests that this individual had conjugate excretion levels very close to the norm for the population. Because the distquan scale was based on an F-distribution with three and 14 degrees of freedom, a score of $\geq$ 3.344 would be considered the statistical cut-off point. Any value $\geq$ 3.344 scored by an individual would indicate that the individual may be not be 'normal', and therefore, needs to be observed closely when being administered medication.

The princomp and Mahalanobis distance values are not the exact absolute values for each individual. This is because the method of derivation for the two methods was different. The princomp method uses an individual's excretion values, for all three pathways, plots them on three axis and then projects the data point down to the first principal component axis to determine what an individual's

princomp value would be. The Mahalanobis distance is an individual's distance, within the data 'cloud', relative to the center of the data 'cloud'. Therefore, two individual's may actually have the same Mahalanobis distance, i.e., same distance from the center of the data set, but when the distance is projected down to the first principal component axis, the individuals would have different princomp values.

Both methods represent a measure of normality, but which method is more accurate and which is to be used as a measure of an index of overall detoxification can not be determined at this time. Clinical studies need to be conducted to ascertain which value, princomp or distance (or both), are more indicative of an individual's index of overall detoxification. It is suggested that values generated in this study be used as markers to alert the clinician to possible problems that may arise when prescribing medications.

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Table 1. Comparison of different statistical methods for developing reference values for urinary conjugate excretion (mmol/24 h) by adult males (n = 18).

Diet	Statistical Method			
	Mean - SD	Mean + SD	P* (16%)	P(84%)
Self-selected Diet (Day 3)				
glucuronides	2.03	3.94	1.98	4.56
sulfoconjugates	1.20	9.37	1.41	12.29
mercapturates	0.10	0.48	0.14	0.71
amino acid conjugates**				
glycine	4.33	5.17	---	---
glutamine	0.98	1.38	---	---
Semisynthetic Diet (Day 2)				
glucuronides	2.25	4.12	2.02	5.00
sulfoconjugates	2.26	6.11	1.79	6.76
mercapturates	0.07	0.24	0.08	0.28
amino acid conjugates				
glycine	4.00	5.42	---	---
glutamine	0.90	1.49	---	---

*P = percentiles (16% & 84% are equivalent to $\pm$ 1 SD)
**Amino acid percentiles could not be determined because n was too small.

TABLE 2. The principal component (princomp) values and Mahalanobis distance (distance) of urinary conjugates determined for 18 male adults consuming a self-selected diet and a semisynthetic diet.

Subject No.	Self-selected Diet		Semisynthetic Diet	
	Princomp	Distance	Princomp	Distance
1	0.704	1.173	0.123	0.004
2	0.562	0.455	1.464	1.890
5	-0.136	0.704	-1.492	0.962
6	-1.334	0.664	0.719	0.361
7	0.537	5.276	2.125	1.825
10	-1.031	2.051	-0.160	0.447
11	-1.016	0.373	-0.145	0.292
12	0.602	0.221	0.204	8.190
13	-0.556	0.230	-0.495	0.196
14	0.129	0.095	0.289	0.076
16	-0.803	0.239	-1.730	1.114
17	-0.050	0.309	0.543	1.668
19	-0.348	0.390	-0.294	0.840
20	-0.124	0.148	0.471	0.855
24	2.204	0.514	0.748	0.293
27	-0.991	0.083	0.022	0.618
28	-0.468	0.976	-0.924	0.441
29	2.117	0.024	-1.468	1.269

RESULTS AND DISCUSSION

The effect of a self-selected and semisynthetic diet on urinary conjugate excretion in 24-h urine samples was determined in 18 male subjects (22-40 y). Subjects were students at Virginia Polytechnic and State University that met certain criteria, i.e., did not smoke or use drugs, were not overweight, and were generally healthy. Subjects consumed a self-selected diet for three days and a semisynthetic/purified diet for seven days, thus subjects served as their own controls. This was done to control for genetic differences between subjects. Conjugate excretion (24-h) was quantified for each subject for each of the 10 study days.

Statistical Analysis System Institute's computer program (SAS, Cary, NC, 1988) was used to conduct the statistical analyses. A MANOVA was done to compare the means of the dietary periods; Helmert transformation for repeated measures was done to determine response to dietary changes.

Sulfoconjugates were found to be excreted in the highest quantity of all the conjugates measured. This was contrary to previously published data. Most of the literature comparing sulfation and glucuronidation indicates that conjugation via sulfation occurs less commonly because of the limited metabolic pool of sulfate, and the smaller number of functional groups capable of forming sulfate conjugates. Other studies have always studied the competition between the two conjugates using a model compound. But the total excretion levels were examined in the present study, which may explain the cause of the discrepancy between the present study and other studies. Additionally, glucuronides can also be excreted in bile and to a higher extent than sulfates (22). Biliary and urinary excretion of glucuronides occurs simultaneously and both excretory pathways are mutually compensatory. Mulder (22) recommends that when looking at both sulfation and glucuronidation both routes of excretion should be followed.

The analytical method used to measure total sulfoconjugates may also have

contributed to the high excretion levels. Sulfoconjugate levels were determined by the difference between total sulfur and inorganic sulfur. Total sulfur was estimated by a wet-digestion method, using fuming nitric acid, which converted any organic sulfur present into sulfate. Sulfate was then indirectly quantified as barium by the precipitation of sulfate as barium sulfate ($BaSO_4$). Thus, any bound sulfur present in the sample (106), e.g., mercapturates (0.14 mmol/24-h); cysteine (0.012 mmol/24-h); taurine (0.068 mmol/24-h); methionine ($6.6x10^{-2}$ mmol/24-h); etc., would also be included in the total sulfate estimate, and therefore, possibly increased the values of the sulfoconjugates as measured by this method. Yet, the levels of these other sulfur-containing compounds should be relatively small and fairly constant from day to day, with the possible exception of mercapturates.

The relationship of the four conjugate types (glucuronides, sulfates, mercapturates, and amino acid conjugates) relative to the total conjugate excretion level and relative to each other are illustrated in **Figure 13**. The Y-axis on the right-hand side of the figure represents the excretion levels for mercapturates, while the Y-axis on the left-hand side of the figure represents the excretion levels for sulfate, glucuronide, amino acid conjugates, and total conjugates. Some of the pathways responded fairly quickly to the change in diet, a change being noticable as soon as the first day of the semisynthetic diet for some of the pathways. Mercapturates responded with about a 50% drop in excretion level from the last day of the self-selected diet to the first day of the semisynthetic diet. This low excretion level ($p<0.0001$) continued for the duration of the semisynthetic diet period. Even though an increase in excretion of mercapturates was observed from day five to seven of the semisynthetic diet, the increase never reached the original excretion levels observed on the self-selected diet. Glucuronide excretion seemed the least affected by diet change. Although there was an initial increase on the first day of the semisynthetic diet that persisted for the rest of the dietary period, it was not significantly ($p<0.05$) different from the excretion levels of the self-selected dietary period. Sulfoconjugate excretion mirrored more the

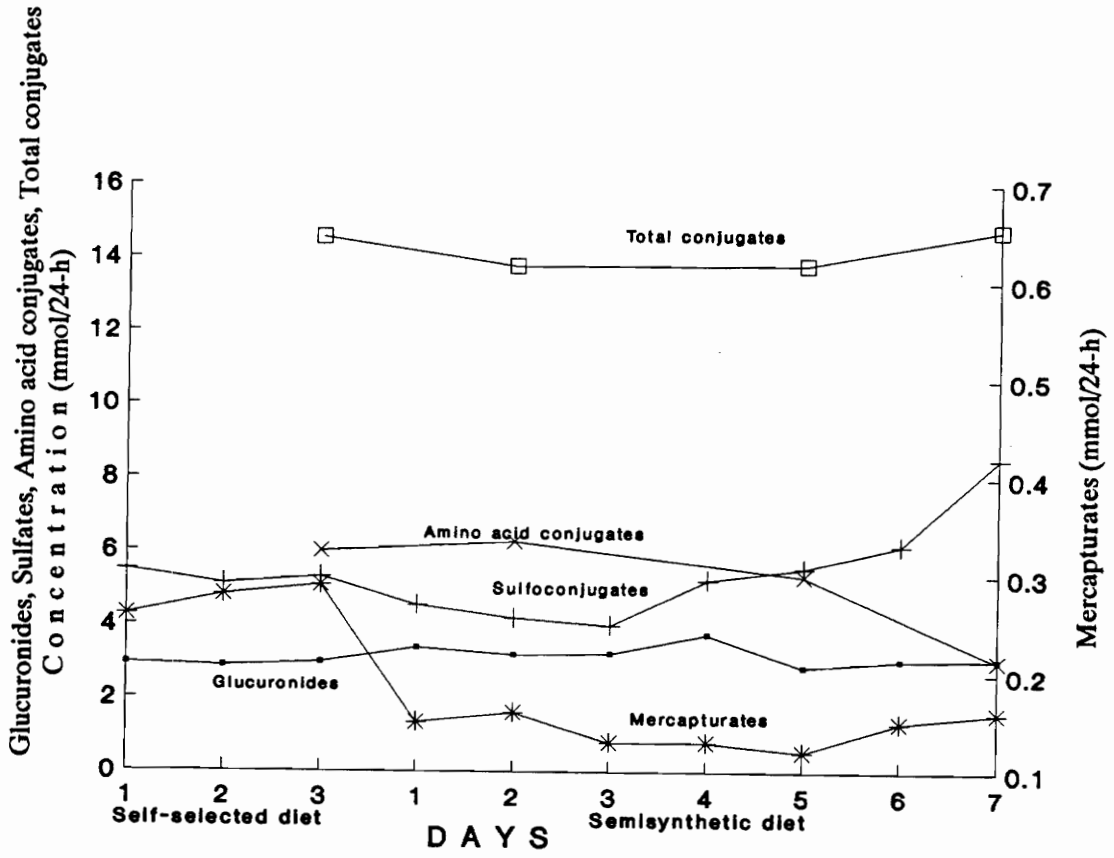

FIG 13. Total conjugate and individual excretion for 18 male subjects consuming a self-selected diet for three days and a semisynthetic diet for seven days. (Amino acid conjugates were measured only on the days indicated, therefore, total conjugate levels are only presented for four days.)

excretion levels of the mercapturates. There was an initial drop of about 14% on day one of the semisynthetic diet and this drop continued until day three. On the fourth day of the semisynthetic diet, the sulfoconjugate excretion started to increase and continued to increase until the seventh day of the semisynthetic diet to a quantity even greater than that attained on the self-selected diet.

Trying to measure excretion levels for all four types of conjugates was generally difficult because our analytical methods looked at categories of conjugates rather than specific conjugate metabolites. This was necessary due to the overall purpose of the study, i.e, to determine a general detoxification profile for individuals. But such methodology has its short-comings. Reactions that apply to an entire category of chemical substances, depend on color development, are more likely to give erroneous results, tend to be less specific, and can introduce more variability to the data. (107). Generally all conjugate levels had very wide ranges and large standard deviations. Although large variability may have been true for all four path-ways measured, the analysis of sulfoconjugates seemed to be most reflective of the wide range of values that can be obtained when using more non-specific methods.

The wide range observed in conjugate levels was of great concern. When repeated measurements of an individual's physiological variable (i.e. urine chemistry) are made over an extended period of time, it becomes difficult to separate long-term analytic or 'laboratory' deviation from the truly physiologic component of the total observed variation. In a normal person, the latter component represents deviations from homeostasis which may arise from short-term trends, cyclic changes, or simply transient events. Therefore, Grubbs' (108) paper on 'Procedures for detecting outlying observations in samples' was used to ascertain the presence of outliers in our data set. Outliers are observations that appear to deviate markedly from other members of the sample in which they appear. Using this procedure it was confirmed that some of the values in the data set could have been outliers. Questions then arise as to whether the outlying observation is merely an extreme indication of the

random variability inherent in the data, or the result of gross deviation from the prescribed experimental procedure, or an error in calculating or recording the numerical value and should it be rejected.

To assist in deciding whether to discard outliers, or to retain them in the data set, the median of the data was compared to the mean (**Fig.14**). The median does not take into account the actual value of each measurement, but only considers the rank of each measurement. Therefore, extremely high or low measurements do not affect the median as much as they affect the mean. As can be seen in **Figure 14.**, the median and the mean of all three conjugate levels were very similar. Therefore, the overall trend seen in the data was real and not due to the effect of outliers.

This study had strong points that are not found in other studies. The approach of studying whole-body detoxification was a more realistic approach for studying conjugate excretion levels. Also, excretion levels were measured for the entire study period giving a more complete picture of how excretion levels changed from day to day. If only certain days had beeb measured, e.g., every third day, or only at the beginning and at the end of the study, the extreme changes in sulfo-conjugate excretion would never have been detected.

Variance in the data was also reduced by introducing a semisynthetic diet. Subjects were put on a semisynthetic diet because previous studies in our laboratory resulted in high inter- and intraindividual coefficients of variation which may have masked any influence due to environmental factors, i.e., diet. A generalized sample variance was determined for the data set to ascertain if variance had actually been reduced (105). When p variables are observed on each data set, the variation is described by the sample covariance matrix. This contains p variances and $\frac{1}{2}p(p-1)$ potentially different covariances. When it is desirable to assign a <u>single</u> numerical value for the variation, the determinant of the covariance matrix is one method of choice for determining this value. Therefore, determinants were calculated for the covariance matrices of the three days of self-selected diet and seven days of semisyn-

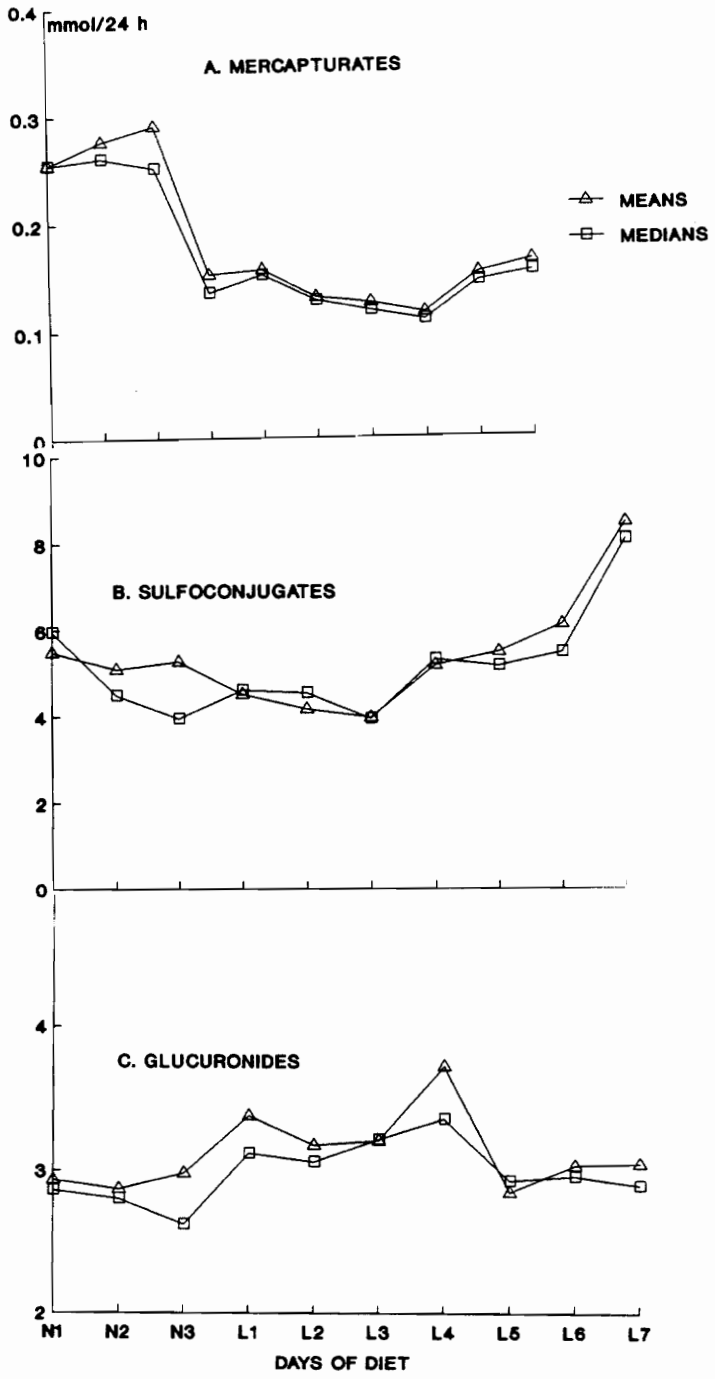

FIG 14. A comparison between the mean and median of three urinary conjugates for 18 male subjects that consumed a self-selected diet for three days and a semisynthetic diet for seven days. (N = self-selected, L = semisynthetic)

thetic diet. This would be equivalent to the generalized sample variance. The log of the determinants was taken to stabilize the variance. The Student's t-test was done on the log transformations of the self-selected diet days and the semisynthetic diet days. The variance of the data set for the semisynthetic diet was found to be significantly lower than that for the self-selected diet ($p<0.002$). The lower variability that occured as a result of the introduction of the semisynthetic diet meant that the data would be more sensitive to experimental manipulation. Thus, if the effect of drugs are to be determined, it might be more beneficial to put subjects on a semisynthetic diet before the introduction of the experimental drug.

SUMMARY AND RECOMMENDATIONS

Eighteen male subjects (22-40 y) were studied to determine the effect of a self-selected diet and semisynthetic diet on conjugate excretion in 24-h urine samples. An overall index of detoxification was also determined. Subjects consumed a self-selected diet for three days and a semisynthetic diet for seven days. Urinary conjugates measured were mercapturates, sulfoconjugates, glucuronides, and amino acid conjugates. Mercapturates and amino acid conjugates were most affected by dietary change, with excretion levels reduced by about 50% during the semisynthetic diet period (0.27±0.11 vs 0.14±0.02 mmol/24-h; 5.99 vs 3.03 mmol/24-h, respectively). Glucuronides were the least responsive to dietary change with no significant difference between the means of the two diet periods (self-selected diet 2.93±0.77; semisynthetic 3.21±0.29 mmol/24-h). Sulfoconjugates were excreted in the greatest quantity of all the conjugates measured. Sulfoconjugates were initially decreased on the semisynthetic diet (5.48±3.86 vs 3.98±1.73 mmol/24-h), but by the fourth day, excretion levels began to increase. This increase could have been due to the presence of vanilla flavoring in the liquid diet. In summary, conjugate excretion was found to be sensitive to dietary changes, with some pathways more responsive to dietary changes than others.

Four methods for developing 'normal' ranges were presented and compared: mean ± SD, percentiles, principal component analysis (princomp), and Mahalanobis distance (distquan) method. The princomp and distquan methods were stressed because they represent a multivariate approach which takes into account the values for all four pathways, and the interactions among the pathways, to develop a single value. This single value would then be representative of an individual's total, or overall, detoxification level relative to the others in the group. The princomp or distquan values could then be used as a 'red flag' by a practitioner, for example, to be on the alert when prescribing to an individual whose values are very different

from the norm.

The results from this study demonstrated that the introduction of a semisynthetic diet caused a decrease in most conjugate excretion levels initially, yet, there occured an increase in excretion that appeared towards the end of the semisynthetic dietary period. Although a pilot study, and other studies reviewed, indicated that seven days of a semisynthetic diet would be sufficient as a wash-out period, it would be interesting to extend this dietary period for another two to three days to see if this rise was transient or a true increase that would continue with time. It would have also have been interesting to see if excretion levels would have increased to the original quantities observed during the self-selected dietary period once subjects were returned to their normal diets.

Mercapturates seem to be the most sensitive to dietary change, therefore, it may not be necessary to analyze for all four pathways when trying to determine the influence of certain dietary treatments. Also, if only mercapturates are being studied, then a wash-out period of only one to two days may be necessary.

Because sulfoconjugates and glucuronides are competing systems and glucuronides are excreted via the urinary and biliary system, it might be more informative to look at biliary excretion of these conjugates in addition to urinary excretion. Although glucuronides appeared not to be affected by diet change, an increase in excretion may have occurred, but via the biliary route, and therefore, not detected.

Very little, if any research, has been done looking at detoxification in females. Hence, a similar study with females instead, would be very informative to see how excretion of these conjugates changes with hormonal fluctuations (i.e., menstrual cycle).

Finally, a similar study could be done using subjects that are on a weight loss regimen and determine how excretion changes with weight-lose. Excessive weight loss would release into the system any xenobiotics that may have been stored in the

adipose tissue and it would be of interest to see what stress would develop in the system with this sudden release of xenobiotics. It might also be of benefit to study the effects of different disease states on these excretion levels. There are a number of situations that can be observed to determine how they affect conjugate excretion rates.

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APPENDICES

APPENDIX A
Selection Criteria Survey Questionnaire

DATE: _____

CODE NUMBER: _____

Please answer all of the following questions accurately and honestly, remembering that any information disclosed will remain confidential.

1. Height: _____ inches

2. Weight: _____ lbs.

3. Have you been hospitalized/outpatient within the last 6 months? If yes, explain.

   a. Yes b. No

4. Are you on a special diet or suffer from food allergies?

   a. Yes b. No

   Specify: _____

   _____

5. Have you used any tobacco products within the last 6 months?

   a. Yes b. No

6. Do you consume alcoholic beverages?

   a. Yes b. No

7. If yes, how often do you consume them? Circle the letter that applies to your amount of consumption.

   a. less than once/day
   b. once/day
   c. 2-3 times/day
   d. more than 3 times/day

8. Do you consume caffeine?

   a. Yes b. No

9. If yes, how often do you consume it? Circle the letter that apples to your amount of consumption.

   a. less than once/day
   b. once/day
   c. 2-3 times/day
   d. more than 3 times/day

10. Have you used any social drugs, such as cocaine, hallucinogens, heroin, marijuana, tranquilizers, etc., within the last 6 months?

    a. Yes b. No

11. Have you taken prescribed or nonprescribed medication within the last 6 months? (i.e steroids; antibiotics; vitamin, mineral, herbal supplements; cough medicine; aspirin; laxatives; antacids; oral contraceptives; or any medication for hypertension, diabetes, seizure, pain, colds, flu, insomnia, etc.)

    a. Yes b. No

12. If accepted for the study, you will be required to not use any drugs. Would you, therefore, be able to resist the use of drugs for a period of 2-4 weeks, unless absolutely necessary?

    a. Yes b. No

13. We are interested in knowing if you are exposed to any chemicals during work hours or while pursuing a hobby. Such chemical 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.

    a. Do you work in a place where you are exposed to any chemicals?

        1. Yes 2. No

        If yes, specify chemical(s):_____

        _____

    b. Do you have a hobby (or hobbies) that expose you to any chemicals?

        1. Yes 2. No

        If yes, specify chemical(s):_____

        _____

14. To the best of your recollection, have you been exposed to organic substances such as pesticides, oil-based paints, etc. for periods of two weeks or more within the last 6 months?

    a. Yes b. No

15. How would you classify your daily exercise regime?

    a. light (sedentary)
    b. mild (1 hr structured activity, i.e., sports, dance, etc.)
    c. moderate (1-2 hr/day, structured activity, i.e, sports, dance, etc.0
    d. vigorous (construction work, etc.)

16. If you answered vigorous in question #15, please describe.

17. Have you had an excessive weight gain/lose within the last 6 months? If yes, explain.

APPENDIX B
Informed Consent Form

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. We also hope to establish what contribution non-nutrients, from our diets, make to an individual's ability to detoxify.

I. SUBJECT REQUIREMENTS

You must be a healthy adult female who is currently not taking any prescribed medication, not under a physician's care, and not working in an area which has high chemical exposure.

II. PROCEDURES

A. Selection Criteria Survey: You will be required to complete a selection criteria survey to determine your eligibility for the study.

B. Food Intake Record: You will be required to keep a dietary record of your food intake for three days. During that time you will be expected to accurately weigh or measure all foods and beverages consumed.

C. Urine Samples: You will be required to collect all urinary excretion produced during a consecutive 2-3 week period. The collection will be submitted to the lab at the end of each 24-hour period. The necessary urine bottles and complete instructions for collection and proper handling will be provided.

D. Blood Samples: Three blood samples of 10-15 ml will be taken on the last day of the normal diet, the last day of the liquid diet, and the last day of the broccoli diet. These blood samples will be used to determine blood cholesterol levels.

E. Diet: You will be required to consume a nutritionally adequate liquid diet, which will be provided to you, for about 2 weeks. The last six to seven days of the liquid diet you will also be required to consume two levels of steamed broccoli, i.e., 250 and 500 grams/day. During this period, you must not consume any other type of food, not take any alcoholic beverages, drugs, etc. Any deviation from this must be reported to the investigators.

III. POSSIBLE RISK

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 for determining the

relative ability of individuals to detoxify xenobiotics. The results of your detoxification ability will be made available to you and this may help your doctor to prescribe a more accurate dosage of a medicine for you, thereby, reducing chances of side effects from the medicine. You will, also, make a savings of two weeks on your food bill, clear your system of caffeine (if you had been a caffeine drinker prior to the study), and be told your blood cholesterol levels.

V. COMPENSATION

For you participation and complete cooperation, you will be offered a cash payment of $100.00, payable upon completion of your involvement in the study.

IV. 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 a right to receive a copy of this document.

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

I have read and fully understood this 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 person is you have any additional questions:

Dr. Ryland E. Webb, principal investigator
Department of Human Nutrition and Foods
Phone Number: 231-6784/5549

APPENDIX C
Instructions for Collection and Handling of Urine

DETOXIFICATION PROFILE STUDY
1991
INSTRUCTIONS FOR COLLECTION AND HANDLING OF URINE

COLLECTION: All urinary excretion produced during the duration of the study must be collected on a 24-hour basis. A 24-hour basis is defined as beginning with the second voiding on day one, through the first voiding on day two. Hence, you will begin collection with the second urinary voiding on Monday morning, collect all subsequent urinary excretions through, and including, the first urinary voiding on Tuesday morning. This 24-hour urine sample must be submitted to the lab on Tuesday morning, when you will be given fresh bottles to begin collecting the second 24-hour sample, and so on. Thus, you must deliver each 24-hour urine sample every day to the lab.

HANDLING: Each urine bottle has been acid washed and autoclaved to prevent contamination. Please handle the bottles on the exterior surface only. If at all possible, we would like to minimize exposure to light and heat; therefore, we request that you refrigerate the bottles at least overnight and keep them in a brown bag. When away from home, try to keep bottles in a cool place (avoid radiators and direct sunlight, etc.). Your cooperation will help to enhance the accuracy of our results and we would be most appreciative.

If you have any additional questions, please don't hesitate to ask us.

APPENDIX D
Daily Food Intake Record

DAILY FOOD INTAKE RECORD

NAME_____

CODE NO._____

DAY OF INTAKE RECORD_____

DATE OF INTAKE RECORD_____

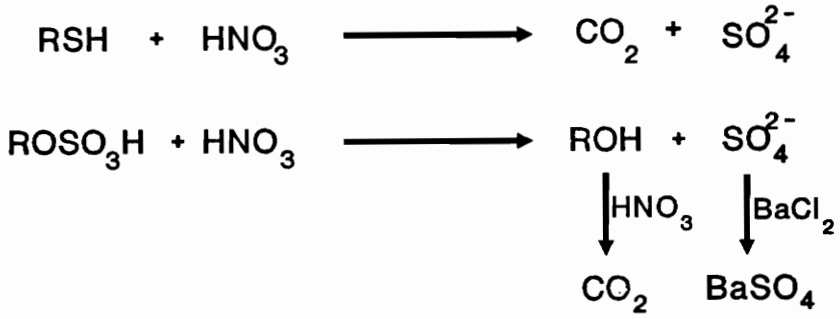
PLEASE RECORD ALL FOOD/DRINK AND AMOUNTS FOR ONE 24-HOUR PERIOD (FROM 6 AM TO 6 AM THE NEXT DAY). IF MIXED DISHES, BREAKDOWN INTO INGREDIENTS IF POSSIBLE (E.G., 1 CUP MACARONI AND CHEESE CASSEROLE: ½ CUP MACARONI, 2 TABLESPOONS CHEESE, ⅓ CUP MUSHROOMS).

FOOD/DRINK	# OF SERVINGS	SERVING SIZE	AMOUNT (g)

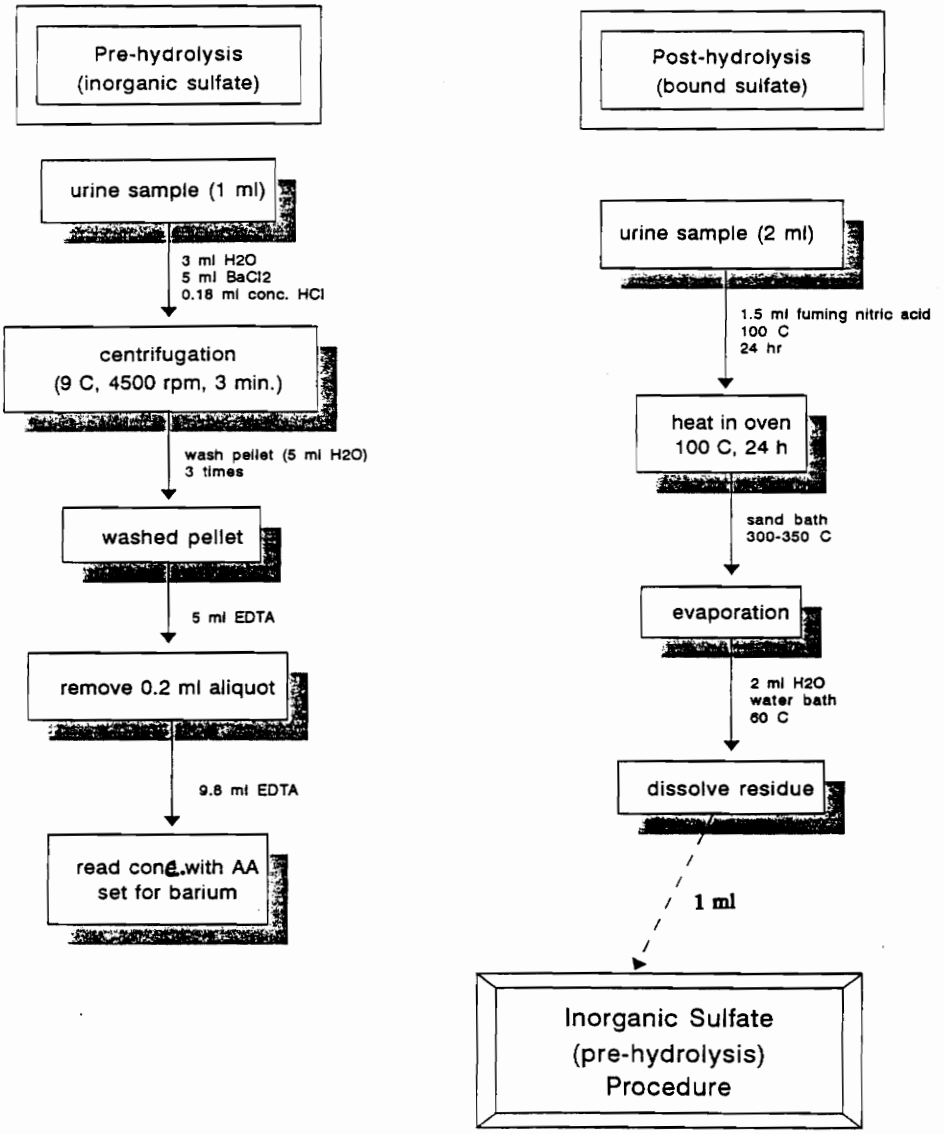
APPENDIX E
Sulfoconjugates Determination

SULFOCONJUGATES DETERMINATION

The method of Lloyd et al. (100) was used to determine urinary sulfoconjugates. Quantification of sulfoconjugates is calculated as the difference between inorganic sulfate before and after hydrolysis with fuming nitric acid. The fuming nitric acid decomposes the sulfur-containing compounds and oxidizes the sulfur released to sulfate. The inorganic sulfate that is produced is percipitated as a barium sulfate pellet, washed three times, dissolved in ethylene diaminetetraacetate (EDTA) solution, and its concentration is then determined by atomic absorption spectroscopy.



A flow chart for this procedure is given:



SULFATE DETERMINATION

A. *Urine preparation*:

1. Mix well 5.0 ml aliquot of urine.

2. Centrifuge for 5 min at 2000 rpm.

B. *Inorganic sulfate analysis*:

1. Pipette of 1 ml aliquots of urine sample into graduated polypropylene centrifuge tubes (15 ml capacity with screw cap, Falcon Brand, Cat. No. C3917-16). Prepare in duplicates.

> [Prepare standards by pipetting 1 ml of standard (Na_2SO_4) (5, 10, 20 ppm) along with the urine samples. Standard does not require duplicate run. These will serve as the standard curve.]

2. Add to these, 3 ml deionized water, 5 ml barium chloride (1.78% (w/v) aqueous $BaCl_2$), and 0.18 ml (5-7 drops) conc. HCl.

> [Solution becomes cloudy. Wait at least 10 min. A white ppt will form at the bottom of the tube.]

3. Centrifuge (DAMON/IEC, DPR-6000) the samples cold (9° C) at 4500 rpm (5,000xg) for 30 min.

4. Discard the supernatant and wash the pellet with 5 ml deionized water, twice. Centrifuge and discard the supernatant with each wash.

> [Supernatant may be poured out with caution. Do not attempt to drain all the liquid because it will disturb the pellet. After the second wash, use a pasteur pipet to remove the last trace of supernatant.]

5. Dissolve the pellet in ethylene diaminetetraacetate solution (Ammonium EDTA) to a final volume of 5 ml.

6. Take a 0.2 ml aliquot of this solution and dilute to 10 ml by adding 9.8 ml of the EDTA solution.

7. Aspirate the sample into the flame of an atomic absorption spectrophotometer (Perkin-Elmer model #2100) which was 'zeroed' to the EDTA solution. Use settings as specified by the manual.

> [Make sure to aspirate the standards before the samples so that the sample concentration can be calculated from the standard curve which is generated by the spectrophotometer's software. Also, check the accuracy of the AA and the position of the burner head by reading a 20 ppm reference standard of Ba.]

C. *Sulfoconjugate analysis*:

1. Take 2 ml urine and place in glass tube (20 x 125 mm, Corning Glass, Corning, N.Y., Cat. No. 9825-20)) with screw caps that have teflon liners. Add 1.5 ml fuming nitric acid into the sample tubes, and hydrolyze for 24 hr at 110_0 C in an oven. Make sure to seal the tubes tightly.

2. Remove the test tubes from the oven and allow to cool to room temperature.

3. Open the tubes and heat them in a sand bath (Fisher Hi-Temp Bath M160) or heating block* at 300-350° C until the nitric acid evaporates.

> [This is the step where most of the sulfate loss might occur. Thus, make sure that the temperature is not too high (390_0 C) so that it destroys the sulfate, but high enough to ensure evaporation of nitric acid in a reasonable time.]

*A heating block was prepared by insulating a roasting pan with vermiculite blocks and placing the pan onto a hot plate. Inside the pan was placed a layer of fine sand. On top of the sand were placed four clay bricks, side-by-side, and another four bricks were placed on this first layer making sure to line-up the holes in the bricks. Then more sand was added around and between the bricks until the bricks were surrounded by sand. Make sure not to pour sand in the holes in the center of the bricks. The hot plate than was turned on to a temperature of about $300\text{-}400^0$ C. When the bricks became hot, the glass test tubes were placed within the holes in the center of the bricks.

4. After evaporation is complete, a white to yellow dried residue will remain at the bottom of the tubes. Add 2 ml of deionized water to this residue.

5. Put in a water bath (60-70_0 C) for 12-15 h to ensure that the residue completely dissolves. Vortex.

6. Pipette 1 ml of this sample into centrifuge tubes.

7. Follow the same procedure as in 'inorganic sulfate analysis' starting with the addition of barium chloride and HCl.

D. Two compounds were used to determine recovery;indoxyl sulfate, an indole derivative, and dehydroisoandrosterone 3-sulfate (DIAS), a steroid sulfate. With each determination of total sulfate analysis, DIAS was run simultaneously to determine the recovery of hydrolysis.

REAGENT PREPARATION:

1. *EDTA solution*: prepare under a fumehood

   a. To 100 ml conc. ammonium hydroxide add 19.07 g potassium chloride.

   b. Dissolve 10 g EDTA, free acid form, to the ammonium hydroxide-potassium chloride solution.

   c. Bring to a final volume of 1000 ml with deionized water, using a volumetric flask.

2. *1.78% (w/v)* $BaCl_2$:

   a.) Measure 1.78 g of barium chloride and bring up to volume with deionized water in a 100 ml volumertric flask.

3. *5 ppm sodium sulfate solution*: for standard curve

   a.) Dissolve 0.1293 g of sodium sulfate in volumetric flask and bring up to 100 ml with water

4. *10 ppm sodium sulfate solution*: for standard curve

   a.) Dissolve 0.2587 g of sodium sulfate in volumetric flask and bring up to 100 ml with water

5. *20 ppm sodium sulfate solution*: for standard curve

   b.) Dissolve 0.5173 g sodium sulfate in volumetric flask and bring up to 100 ml with water

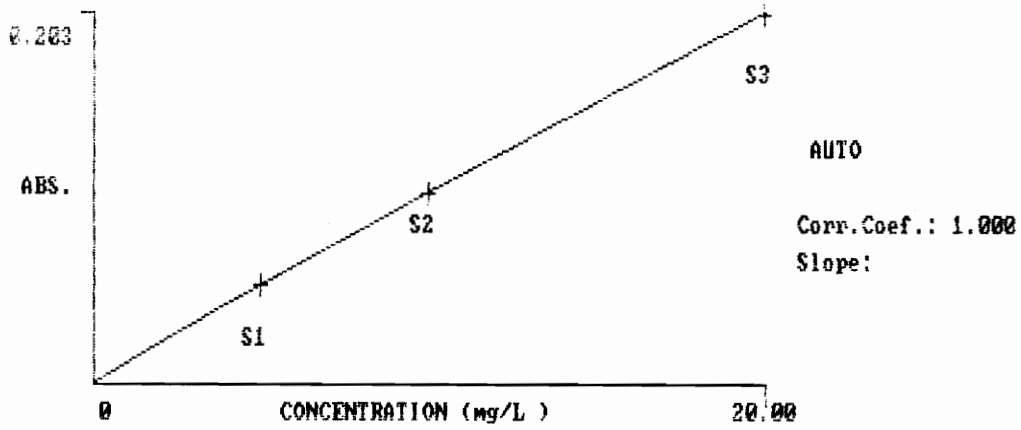
6. *Dehydroisoandrosterone-3-sulfate (DIAS)*: $C_{19}H_{27}O_5SNa$
   MW = 390.5; SO_4 = 96 MW (24% of DIAS)

   a.) Dissolve 1 g of DIAS in deionized water and bring up to 100 ml in volumetric flask. This gives 1 g/100 ml = 10,000 μg/ml = 10,000 ppm DIAS; 2,400 ppm SO_4

   b.) This solution will be further diluted by 250x before it is measured with the AA spectrophotometer, ∴ 2,400 ppm ÷ 250 = 9.6 ppm.

Atomic Absorption Spectrophotometer Settings (Perkin-Elmer M2100)

Wavelength (nm): 553.5	Slit (nm): 0.2
Lamp: Barium cathode	Lamp current (nm): 16
Integration Time (sec): 3.0	Read Delay (sec): 1.0
Oxidant: Nitrous oxide (N_2O)	Oxidant Flow (L/min): 5.3
Fuel: Acetylene (C_2H_2)	Fuel Flow (L/min): 6.5
Energy: 70	

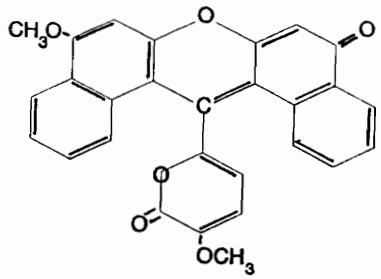


Standard curve for sulfate determination using 5, 10, 20 ppm sodium sulfate standards

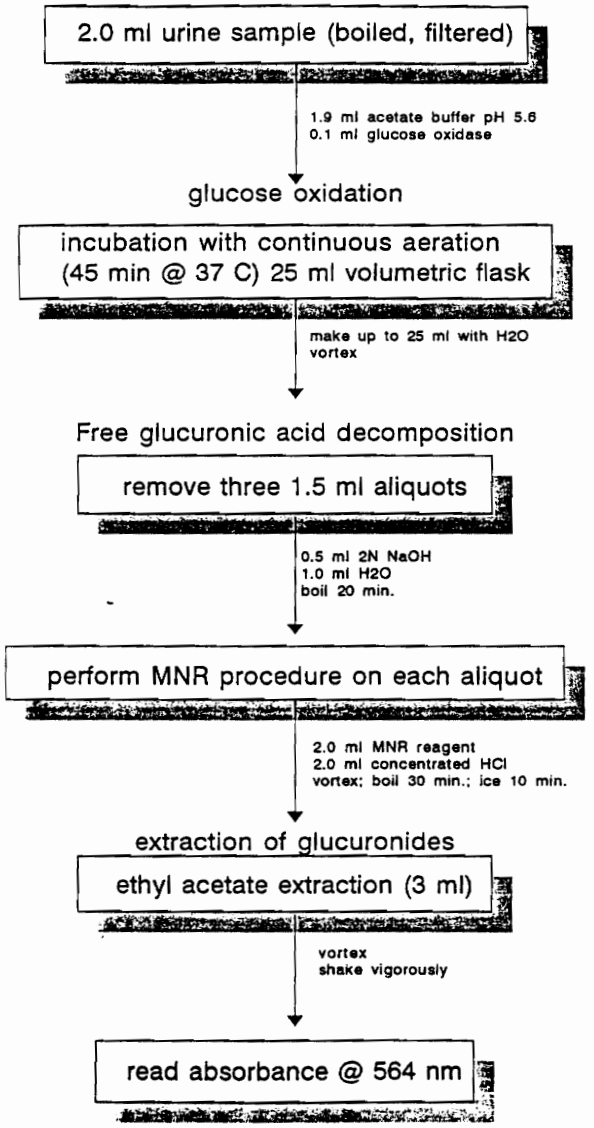
APPENDIX F
Conjugated Glucuronic Acid Determination

CONJUGATED GLUCURONIC ACID DETERMINATION

The modified naphthoresorcinol (MNR) method of Mazzuchin et al. (101) was used to quantify urinary conjugated glucuronic acid. Glucose interference is selectively eliminated with glucose oxidase enzyme. This permits the determination of conjugated glucuronic acid even though the urine may contain high glucose concentrations. Free glucuronic acid is decomposed with NaOH to a product insensitive to the MNR reagent. Strong acid conditions then liberate the glucuronic acid from the conjugate. The liberated glucuronic acid then reacts with the MNR reagent to produce a compound which has a characteristic pinkish - bluish color. The naphthoresorcinol reaction is believed to involve a partial dehydration of the uronic acid to the lactone and then conjugation with the reagent to produce the following compound;



A flow chart for this procedure is given:



GLUCURONIDE DETERMINATION

A. *Urine preparation*:

1. Mix well 5.0 ml aliquot of urine.

2. Boil for 20 min in a 100^0 C water bath. Cool.

3. If ppt. appears, transfer to conical graduated polystyrene centrifuge tubes (Fisher Brand, Cat. No. 05-527-45) and centrifuge (DAMON/IEC, HN-SII) for 5 min at 2000 rpm (1,300xg).

4. Decant supernatant and use for the rest of the procedure.

B. *Glucose oxidation*: to remove glucose interference by converting free glucose to gluconic acid which is insensitive to the NR reagent:

1. Into a 25 ml volumetric flask, add 2.0 ml of the boiled urine. Then add 1.9 ml of .1 M acetate buffer (pH 5.6), and 0.1 ml glucose oxidase Type V (G-6891, approx. 1,000 units/ml, Sigma Co.). Prepare a blank, using glucose solution (l mg glucose/1 ml water) instead of urine, using the same procedure as for the urine.

2. Incubate the volumetric flasks in a water-bath at 37^0 C for 45 min. Continuously aerate the mixture by blowing air into the flask, using a pasteur pipet attached to a manifold connected to the air line in the laboratory hood air valve. The air needs to be filtered through fiber glass.

3. After incubation, bring the volume up to 25 ml with deionized water. Mix well the contents.

C. *Decomposition of free GA to produce a product which is insensitive to NR reagent:*

1. Remove three, 1.5 ml aliquots of the diluted urine and blank, and transfer to glass test tubes, with screw caps lined with teflon, containing 0.5 ml of 2N NaOH and 1.0 ml deionized water.

2. Boil this mixture for 20 min. Allow to cool to room temperature in an ice bath.

D. *MNR procedure: glucuronide determination*:

1. After boiling, add 2.0 ml MNR reagent and 2.0 ml concentrated HCl.

   [The reaction product formed is pink-bluish in color and stable for about 1 hour]

2. Vortex, then boil for 30 min. After boiling, cool on ice for 10 min.

3. Vortex again, and then add to the test tubes 3.0 ml ethyl acetate and shake vigorously, by hand, for 15 sec.

   [Ethyl acetate extracts the color product from the aqueous layer. It is necessary to shake vigorously in order to assure complete extraction of the color product.]

4. Pipette the acetate layer into a glass cuvette and read the absorbance at 564 nm against the blank.

5. Determine the concentration of conjugated glucuronic acid from a standard curve.

6. Repeat any sample where the replicates differ by >5%.

E. *Standard curve*:

Phenolphthalein glucuronic acid (P-Gibe concentrations (ug/ml) to be used to develop standard curve using spiked urine samples:

0; 20; 40; 50; 80; 100; 160; 200; 250 μg/ml

1. Into a 25 ml volumetric flask add 2 ml of urine, 1.9 ml 0.1 M acetate buffer (pH 5.6), and 0.1 ml glucose oxidase Type V. Prepare two other 25 ml volumetric flasks in a similar manner. Prepare a blank by adding buffer and enzyme to 1 ml of glucose solution (1 mg/ml) and 2 ml deionized water instead of 2 ml urine.

2. Follow the rest of the glucose oxidase step.

3. Remove, and place in glass test tube with teflon lined screw caps, three 1.5

ml aliquot of urine from step #2 and spike with 1 ml of P-Gide standard solution (spiked sample). Repeat for all eight P-Gide standards. Prepare triplicate unspiked samples by replacing the P-Gide spike with 1 ml deionized water and prepare a blank by replacing the urine with 1.5 ml of blank prepared in #1.

4. Perform the GA decompostion step.

5. Perform MNR procedure.

6. Develop a standard curve of absorbancy vs μmoles of P-Gide.

REAGENT PREPARATION:

A. *Preparation of Modified naphthoresorcinol reagent (MNR)*:

1. In a 100 ml volumetric flask, dissolve 400 mg of naphthoresorcinol (NR) in 80 ml deionized, distilled water.

2. Add, drop-wise, 0.5N sodium hydroxide until the pH is between 8 and 8.5.

[The solution should turn deep yellow.]

3. Let this solution stand for 15 min. Then add, while stirring with a magnetic stirrer, 10% (w/v) meta-phosphoric acid from a pasteur pipet until pH 2-2.5 is obtained.

[Solution should turn light yellow. Acidification helps to stabilize the reagent.]

4. Add sodium bisulfite (200 mg) and adjust volume to 100 ml with deionized water.

[Bisulfite also stabilizes the reagent.]

5. Filter the solution twice, using vacuum, and refrigerate at 0-5^0 C until ready to use. Protect from light by wrapping volumetric flask with aluminum foil.

6. The reagent will remain stable for 3-7 days.

2. *0.1 M Acetate Buffer:* pH = 5.6

   a.) Prepare 0.2 M glacial acetic acid by measuring 1.155 ml of the acid using a pipetteman, and bring up to 100 ml with deionized water in a volumetric flask.

   b.) Prepare a 0.2 M sodium acetate solution by measuring 13.60 g of sodium acetate and bring up to 500 ml with deionized water.

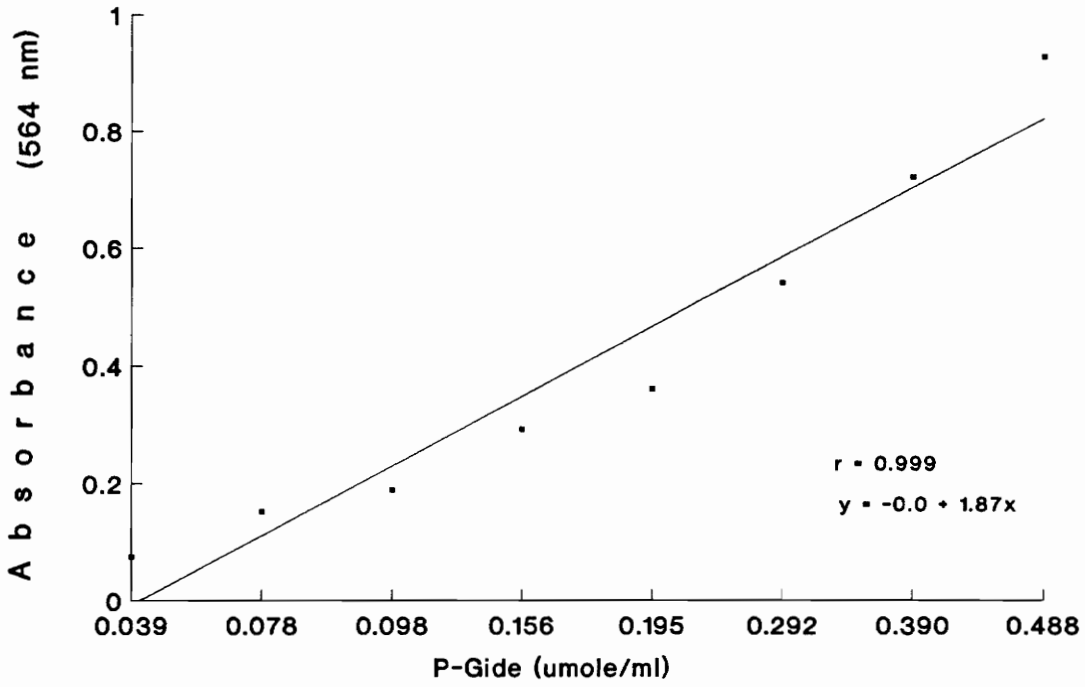
   c.) Mix 48 ml of solution 'a' with 452 ml of solution 'b' and bring up to 1000 ml. Adjust to pH 5.6 by adding either 'a' or 'b', accordingly.

3. *0.5 N NaOH*:

   a.) Measure 50 ml of 1 N NaOH and bring up to 100 ml with deionized water in volumetric flask.

4. *10% (w/v) Meta-phosphoric acid*:

   a.) Measure 10 g of phsophoric acid and bring up to 100 ml with deionized water in a 100 ml volumetric flask.

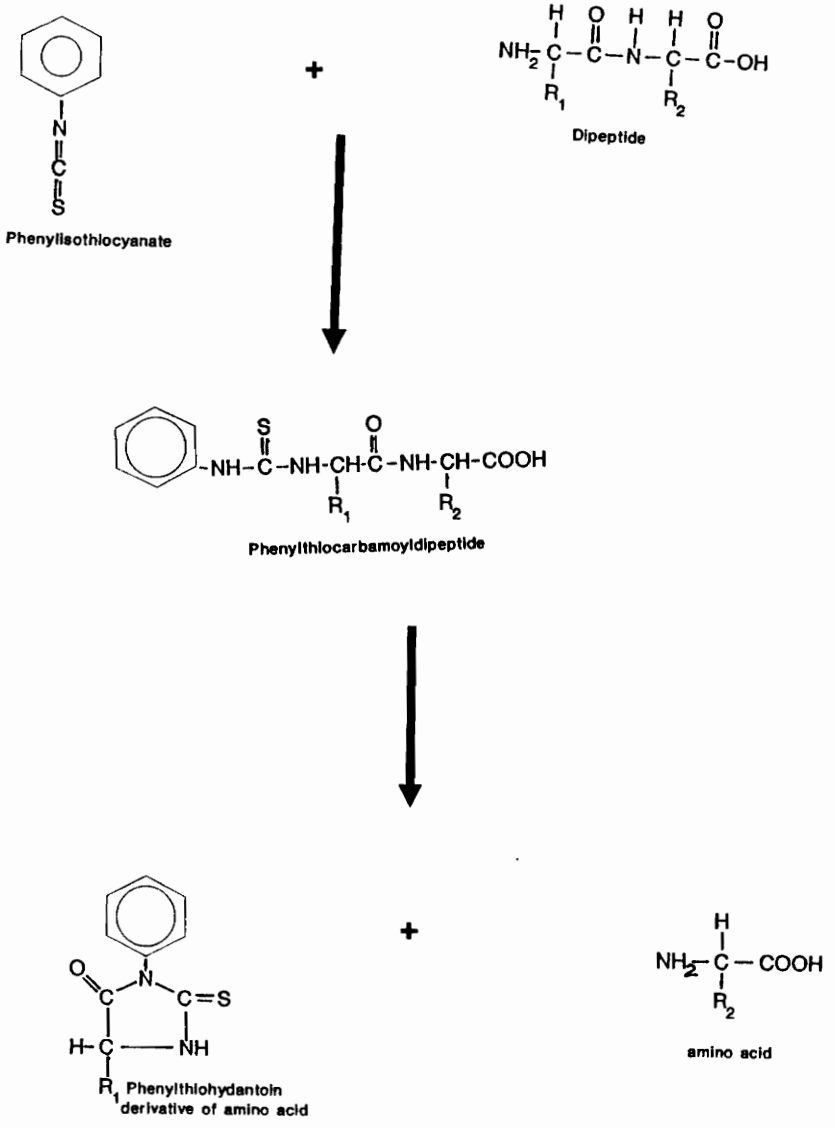


Standard curve for glucuronide determination using urine spiked with P-Gide

APPENDIX G
Amino Acid Conjugates Determination

AMINO ACID CONJUGATES DETERMINATION

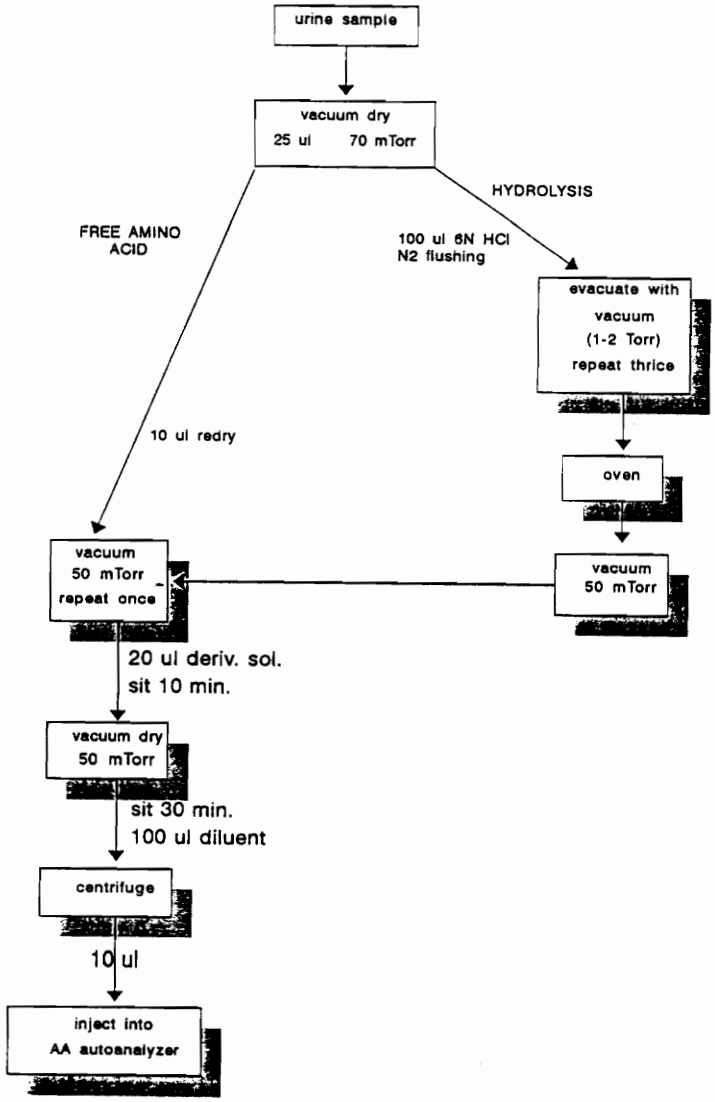
The method of Bidlingmeyer et al. (102) was used to determine urinary conjugated glycine, glutamine, and taurine. This method of amino acid analysis is based on the use of the Edman reagent, i.e., phenylisothiocyanate (PITC). PITC is used for the quantitative precolumn derivatization of free amino acids. PITC derivatization results in phenylthiocarbamyol (PTC) derivatives which then can be separated by HPLC.



Since the urinary excretion of peptides and compounds containing free amino groups other than conjugated amino acids is negligible in the daily excretion of urine in a healthy adult population, it is assumed that the contribution from theses compounds to measurements of urinary glycine and glutamine is not significant. Moreover, any substance, including peptides, which has a molecular weight greater than 10,000 was removed by ultrafiltration.

The quantification of the conjugated compounds was done by difference: (amount of amino acid before hydrolysis) minus (amount of amino acid after hydrolysis). Thus, glycine conjugates were determined by subtracting free, prehydrolysate urinary glycine from total, posthydrolysate glycine. Glutamine conjugate determination was calculated by subtracting free, prehydrolysate urinary glutamine and glutamic acid from total posthydrolysate glutamic acid, since free glutamine converts to glutamic acid upon hydrolysis.

A flow chart for this procedure is given:



AMINO ACID CONJUGATE DETERMINATION

A. *Urine preparation*:

1. Dilute the urine 1:1 with internal standard solution (200 μL norluecine).

2. Filter the sample through a Millipore filtration device (ultrafree MC-10000 NMWL, low binding cellular filter).

3. Spin at 1,500 g for 15 min (preferably in a refrigerated centrifuge).

4. Collect the filtrate and place it in labeled micro-vials. Freeze (-20^0 C) until derivatization.

B. Free amino acid analysis:

1. Place the filtered urine (25 ul), in duplicates, in a 6 by 50 mm tube. Place tubes into a reaction vial and place in the vacuum station. Dry to 70 mTorr.

> [The samples should be placed in a dessicator jar in the freezer if they are to be run at a later date. Hydrolyze the hydrolysis samples previous to derivatizing the free amino acid smaples so they can be run at the same time.]

2. Add 10 μL of Redry solution to each standard and sample tube, vortex, place in a reaction vial and dry to 50 mTorr. Let sit at 50 mTorr for 15 min.

> [Make up the derivitization reagent during this period. It must sit for at least 5 min after mixing it.]

3. Repeat step 2.

4. Add 20 μL 0f derivitization reagent to each tube. Vortex the tubes, place them in the reaction vials and place the reaction vials in the vacuum station <u>without</u> opening the vacuum ports. Let sit 10 min. Open the ports and dry to 50 mTorr. Let sit at 50 mTorr for 30 min.

> [Samples may be stored for up to 24 h after derivitization in a dessicator jar in the freezer (-20^0 C).]

5. Using a Hamilton syringe, add 100 μL of diluent to each sample tube and vortex. Place the sample tubes in the small microcentrifuge (Speed-Vac) and spin for 30 s on low (1,200 rpm).

6. Remove the supernatant (with a 9" pasteur pipette) to a limited volume insert and place it in the appropriate WISP vial.

7. Using forceps, place Teflon septa in the open-top vial caps and screw the tops on the WISP vials. Place the vials in the refrigerator until ready to run.

[Samples can be stored this way for up to 24 h, but it is not recommended.]

8. In running the program:

a) Use a "physiological" program.
b) The volum of extract should be "1" in the "Edit Queue-wide Parameters" section of the Sample Queue.
c) Enter the amino acid concentrations in the concentration table of the Sample Queue (check the card which comes with the standard).
d) Set the run time for 88 min.
e) Make sure the gradient is correct.
g) The "dilution factor' in the Sample Queue should be 0.08.
g) The "amount" in the Sample Queue should be 1.
h) The "injection volume' should be 10 μL.

[Refer to section #1, A.A. Analyzer instructions: setting up the analyzer for a run.]

C. *Hydrolyzation: peptide analysis*

1) Follow the same procedure up to step #2 of part B as in the free amino acid analysis.

2) Add 100 ul of 6 N HCl with 1% phenol to the bottom of the reaction vessel containing the individual sample tubes. Then purge the reaction vessel with nitrogen and cap.

[This allows the use of HCl vapor for hydrolsis instead of HCl liquid. Be careful not to get any inside the sample tubes.]

3) Place the vial in the vacuum station and pull a slight vacuum (1-2 Torr) for about 20 s or until you see the HCl start to boil. Close the valve and remove it from the vacuum station.

4) Purge the vessel with nitrogen using the small hole on the top and the valve (do not unscrew the cap). Quickly close the valve.

5) Repeat steps 2 & 3 twice more.

6) Place reaction vessel in oven (105-112^0 C) for 20 to 24 h.

7) After hydrolysis, dry samples again using vacuum to 50 mTorr.

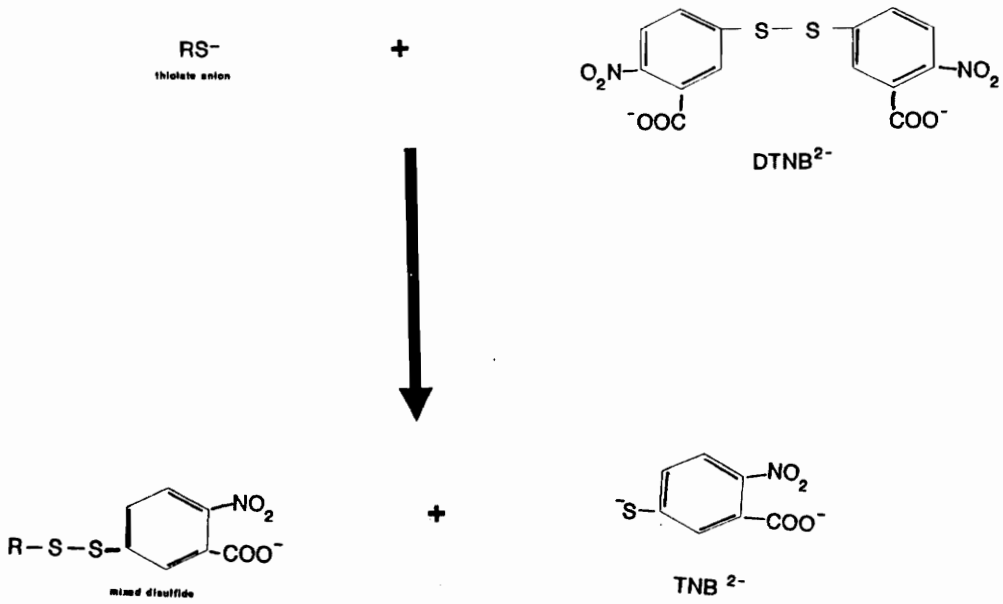
8) Samples are now ready to derivitize as in the free amino acid procedure.

WEAR LATEX GLOVES AT ALL TIMES TO AVOID CONTAMINATION OF SAMPLES

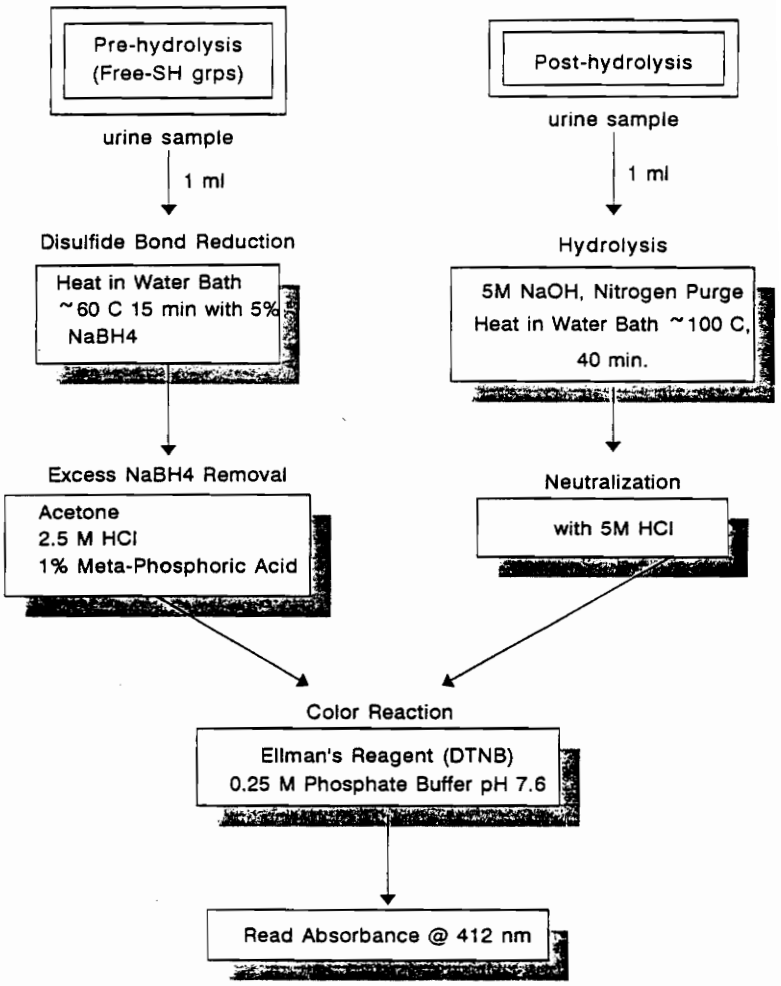
APPENDIX H
Mercapturic Acid Determination

MERCAPTURIC ACID DETERMINATION

A modified method by Seutter-Berlage (103) was used to determine quantities of urinary mercapturates, using Ellman's reaction. Generally, the basic procedure will involve NaOH hydrolysis of the thioether bond under nitrogen gas. A thiol-disulfide exchange reaction between the reduced sulfhydryl compound and the Ellman reagent, dithiobisnitrobenzoic acid (DTNB), generates a highly colored dianion TNB^{2-} for indirect spectrophotometric analysis. Non-hydrolyzed,, reduced samples (reduction is done by using $NaBH_4$) were used to determine free sulfhydryl and disulfides naturally present in the urine. The urinary mercapturic acid levels were then determined by difference.



A flow chart for this procedure is given:



MERCAPTURATE DETERMINATION

A. *Urine preparation*:

1. Mix well 5.0 ml aliquot of urine.

2. Boil for 20 min in a 100^0 C water bath. Cool.

3. If ppt. appears, transfer to centrifuge tubes, centrifuge for 5 min at 2000 rpm (1,300 x g; DAMON/IEC, HN-SII).

4. Decant supernatant and use for the rest of the procedure.

B. *Determination of free sulfhydryl groups (pre-hydrolysis)*:

1. Start heating a water bath to a temperature of 60° While waiting for the water bath to reach the desired temperature, make a fresh solution of 5% $NaBH_4$ (5 g/100 ml of deionized water => 1.3 M).

> [The sodium borohydride solution should be made in a graduated test tube which is placed in a beaker and surrounded by crushed ice. Let the solution stand for at least 15 min. This reduces the amount of effervescence that occursin step #2.]

2. Mix 1 ml of clear urine, from Part A, with 1 ml of the freshly prepared 5% $NaBH_4$ solution in stoppered polypropylene test tubes (95x16.8 mm, 13 ml; Cat. No. 55.518, Sarstedt Inc., Princeton, N.J.). Also prepare a blank, in the same manner as the urine sample, using l ml of deionized water instead of urine. Prepare all samples in triplicate. Stopper tightly all test-tubes.

> [The purpose of this step is to reduce any -S-S- groups which may be in the urine.]

3. Heat in a water bath for 15 min. Keep the test tubes stoppered to reduce oxidation of the free -SH groups.

4. After 15 min remove test tubes and cool in an ice-bath for 10 min. Also put acetone and 2.5 M HCl in an ice-bath (these will be used to destroy excess $NaBH_4$).

5. To destroy excess $NaBH_4$:
   a) add 1 ml acetone to test tubes - shake and wait for 5 min.

   b) add 1 ml 2.5 M HCl and wait 10 min. Shake and make sure that there is no effervescence. If effervescence does occur, this signifies that incomplete destruction has taken place (add more HCl if necessary).

   c) Add .2 ml 1% (w/v) metaphosphoric acid, mix and wait 2 min.

   [Complete destruction of sodium borohydride must occur because it also reacts with Ellman's color reagent. Decomposition must be done slowly and carefully in a fume hood because a poisonous gas (BH_3) is evolved and foaming may also occur. Addition of acetone before HCl helps to reduce the amount of foaming that occurs.]

6. Spectrophometric reading:
   a) set spectrophotometer to 412 nm
   b) *for color blank & urine sample*, put into a cuvette:
      2.00 ml .25 M (pH 7.60) phosphate buffer
      0.30 ml Ellman's reagent (DTNB)
      0.75 ml sample from test tubes (i.e., urine samples or blank)

   c) *for urine blank*, put into a cuvette:
      2.00 ml 0.25 M (pH 7.6) phosphate buffer
      0.30 ml deionized water
      0.25 ml urine sample from test tubes

   d) cover the cuvettes with cuvette cap squares (Fisher Brand, Cat. No. 14-385-999) and invert before read absorbancy

   e) wait 10-20 min

   f) zero the spectrophotometer with the color blank prepared in section 6b

   g) read the absorbance at 420 nm

   h) subtract the urine blank (6c) for the sample blank (6b) to

substract color due to the urine being tested; this is your reading for the urine sample

i) if replicates differ by >5%, repeat these samples

[Timeing needs to be the same for all samples because the characteristic yellow color continues to increase in intensity with time.]

C. *Determination of free-SH groups (after hydrolysis)*:

1.) Aliquot 1 ml of fresh urine sample into a polypropylene test tube with screw cap and add to it 1 ml 5 M NaOH.

[The NaOH cleaves the -S- bonds of the mercapturic acid, as well as the -S-S- bonds, to liberate a free -SH group.]

2) Bubble nitrogen through this solution for 15 s and close tightly to prevent oxygen entering.

3) Heat in boiling water bath (100 - 105° C) for 40 min. Cool in an ice bath for 10 min.

4) Neutralize the NaOH with 1 ml 5 M HCl. Mix and wait for 10 min.

[Be careful when adding HCl. An excess of the acid will interfere with the color development, if the pH is too low, and can cause large variability between replicates.]

5) Color reaction and spectrophometric reading: repeat step 6 in part A.

Two compounds were used to determine recovery:
N-acetyl cysteine (a free sulfhydryl group),
phenyl mercapturic acid (conjugated acid).

D. *Standard curve*:

1. N-acetyl cysteine (NAC) is the reference compound used for the development of separate standard curves for the pre-hydrolysis and post-hydrolysis determination. The standard curves were developed with spiked urine samples using the following concentrations:

2. The concentrations of NAC used to develop the standard curve were:

0.05; 0.10; 0.20; 0.40; 0.50; 0.60; 0.80; 1.0; 1.5 μmol.

3. Mix 0.5 ml of NAC standard of each of the above concentrations into separate test tubes. Add to these 0.5 ml of a urine sample that has been randomly selected from the urine samples collected (spiked samples). Prepare, also, 0.5 ml urine and 0.5 ml deionized water (unspiked sample).

4. Follow the same procedure for pre-hydrolysis determination. Subtract reading of unspiked sampes from spiked samples, then plot absorbency vs concentration of NAC.

5. Repeat steps 1-4, but follow the procedure for post-hydrolysis determination.

6. Phenolmercapturic acid was used to varify the hydrolysis step.

REAGENT PREPARATION

1. *Phosphate buffer solution*: 0.25 M, pH = 7.60

a.) Na_2HPO_4 - sodium phosphate, dibasic; MWt = 141.96
Catalog No. S-374, Fisher Sci.

dissolve 35.49 g in deionized water and make up to 1000 ml

b.) NaH_2PO_4 - sodium phosphate, monobasic; MWt = 137.99;
Catalog No. S-369, Fisher Sci.

dissolve 17.25 g in deionized water and make up to 500 ml

c.) Add the monobasic solution to the dibasic solution, while stirring with a stirring rod, until reach pH = 7.60 (Final pH need not be very exact, because the color reaction occurs within a pH range of 6-8).

d.) put in refrigerator

2. *5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)*: Ellman's reagent

a.) measure 40 mg of DTNB and;

b.) measure 1.12 g sodium citrate

c.) dissolve and make up to volume with deionized water in a 100 ml volumetric flask

d.) store at 4^0 C. Solution will remain stable for 13 weeks

3. *Meta-phosphoric acid*:

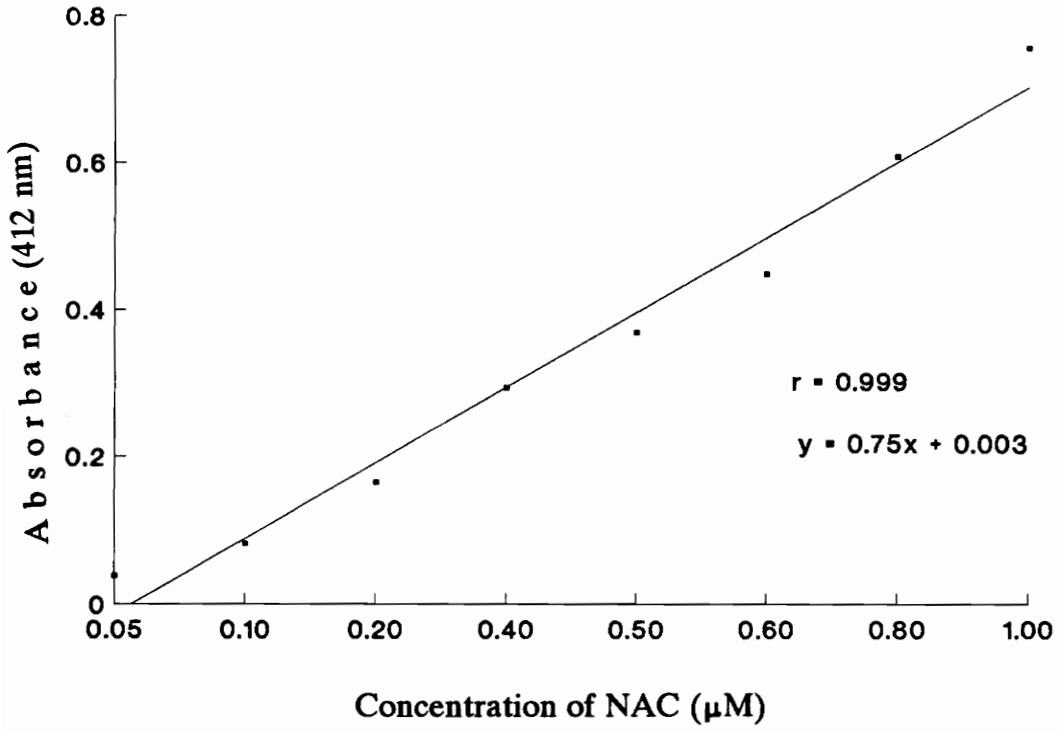
a.) measure 1 g of m-phosphoric acid

b.) dissolve and make up to 100 ml with deionized water in volumetiric flask

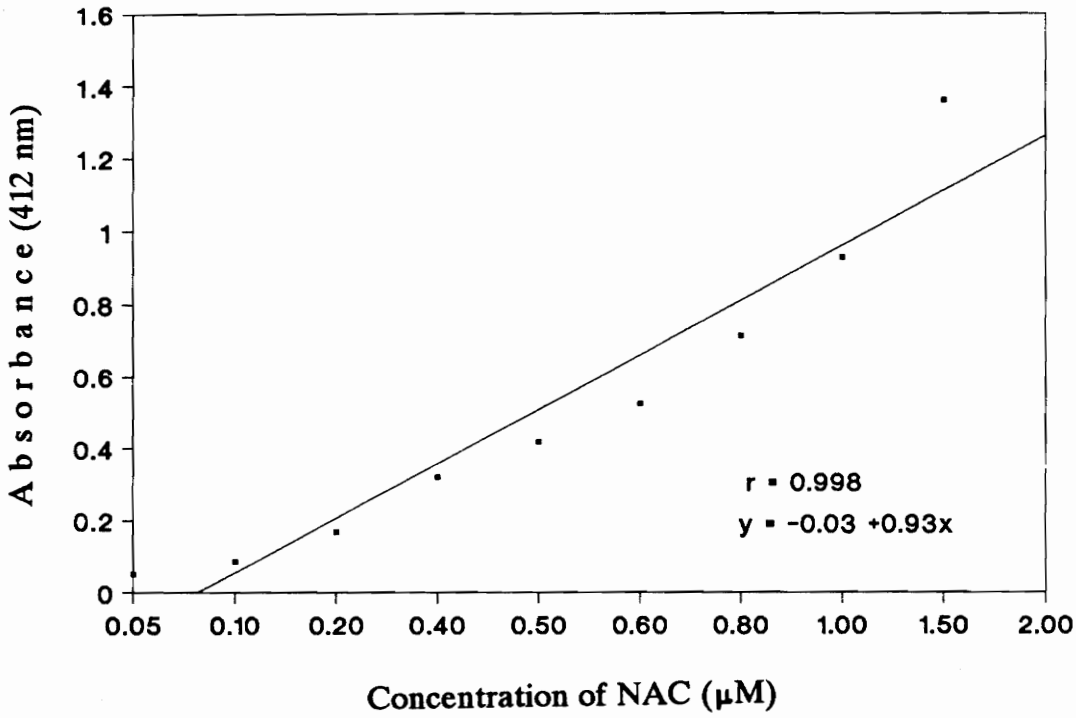
c.) can be kept at room temperature

4. *N-acetyl cysteine (NAC) stock solution*: 100 mM (100 μmol/ml = 0.1 M)

a.) dissolve 1.64 g NAC and make up to volume with deionized water in 100 ml volumetric flask.



Standard curve for per-hydrolysis mercapturate determination using N-acetyl cysteine as the standard



Standard curve for post-hydrolysis mercapturate determination using N-acetyl cysteine as the standard

APPENDIX I
Daily Weight Change of Subjects

Daily weight change of the 18 subjects, expressed as kg, during the experimental period

SUBJECT NO.	N1	N2	N3	L1	L2	L3	L4	L5	L6	L7	WEIGHT CHANGE (kg)
1	74.90	74.20	74.50	75.40	74.50	74.30	74.60	74.70	74.40	75.10	+0.20
2	83.10	82.60	83.00	82.00	81.90	83.50	82.70	82.30	82.50	82.20	-0.90
5	98.00	98.20	98.30	97.00	96.40	97.00	97.20	97.20	96.40	96.00	-2.00
6	69.50	69.70	69.50	68.95	69.05	69.60	69.40	69.20	69.60	68.50	-1.00
7	79.10	79.45	79.50	79.40	79.40	78.50	78.90	78.30	78.20	78.20	-0.90
10	94.50	94.25	94.40	93.80	93.80	93.80	93.90	93.20	93.30	93.20	-1.30
11	76.00	76.60	75.30	74.50	74.50	75.70	76.00	76.40	77.20	76.10	+0.10
12	76.10	77.20	76.00	75.40	74.60	75.00	75.70	74.00	75.40	76.20	+0.10
13	65.90	67.00	65.70	65.80	65.70	65.90	65.70	65.50	66.20	65.80	-0.10
14	96.10	96.10	95.70	94.50	94.50	94.50	94.50	94.30	94.25	94.35	-1.75
16	58.90	58.90	59.10	58.60	58.60	58.60	57.90	58.70	58.40	58.00	-0.90
17	68.50	68.70	68.40	68.40	68.40	67.70	67.30	66.80	67.70	67.60	-0.90
19	86.60	86.50	86.40	86.70	87.30	87.50	87.30	86.60	86.20	86.10	-0.50
20	66.30	66.00	65.80	65.40	64.90	65.30	65.20	65.50	65.30	65.40	-0.90
24	77.20	76.60	76.80	76.70	75.40	76.10	75.95	76.65	77.15	76.30	-0.90
27	74.40	73.40	73.40	73.40	73.40	73.50	73.60	74.15	74.20	75.10	+0.70
28	69.15	69.15	69.30	68.50	68.80	70.00	70.60	69.70	69.30	69.60	+0.45
29	72.20	73.10	73.20	71.70	70.80	71.15	70.25	71.05	71.30	71.00	-1.20
											$\bar{x}$=-0.65

N1-N3 = Self-selected diet period (day 1 - day 3)

L1-L7 = Semisynthetic diet period (day 1 - day 7)

(+) = weight gain

(-) = weight loss

APPENDIX J
Mean Nutrient Intake of 18 Subjects for Two Different Dietary Periods

The mean nutrient intake of 18 subjects for two different dietary periods.

Diet Period	Kilocalories	% Kilocalories			Dietary Fiber
		Carbohydrate	Protein	Fat	
Self-selected (mean of 3-d)	2811	54	15	31	22
Semisynthetic (mean of 7-d)	2700	57	14	29	34

APPENDIX K
Repeated Measures Analysis with Helmert Transformation for Mercapturates

General Linear Models Procedure

Number of observations in data set = 18

General Linear Models Procedure
Repeated Measures Analysis of Variance
Repeated Measures Level Information

Dependent Variable	MERN1	MERN2	MERN3	MERL1
Level of LIQUID	1	2	3	4
Dependent Variable	**MERL2**	**MERL3**	**MERL4**	**MERL5**
Level of LIQUID	5	6	7	8
Dependent Variable	**MERL6**	**MERL7**		
Level of LIQUID	9	10		

General Linear Models Procedure
Repeated Measures Analysis of Variance

Manova Test Criteria and Exact F Statistics for
the Hypothesis of no LIQUID Effect
H = Type III SS&CP Matrix for LIQUID E = Error SS&CP Matrix

S=1 M=3.5 N=3.5

Statistic	Value	F	Num DF	Den DF	Pr > F
Wilks' Lambda	0.090527	10.046	9	9	0.0010
Pillai's Trace	0.909473	10.046	9	9	0.0010
Hotelling-Lawley Trace	10.04645	10.046	9	9	0.0010
Roy's Greatest Root	10.04645	10.046	9	9	0.0010

General Linear Models Procedure
Repeated Measures Analysis of Variance
Univariate Tests of Hypotheses for Within Subject Effects

Source: LIQUID

DF	Type III SS	Mean Square	F Value	Pr > F	Adj Pr > F G - G	Adj Pr > F H - F
9	0.70256911	0.07806323	10.39	0.0001	0.0002	0.0001

Source: Error(LIQUID)

DF	Type III SS	Mean Square
153	1.14907849	0.00751032

Greenhouse-Geisser Epsilon = 0.2344
Huynh-Feldt Epsilon = 0.2685

General Linear Models Procedure
Repeated Measures Analysis of Variance
Analysis of Variance of Contrast Variables

LIQUID.N represents the contrast between the
nth level of LIQUID and the mean of subsequent levels

Contrast Variable: LIQUID.1

Source	DF	Type III SS	F Value	Pr > F
MEAN	1	0.11788210	23.90	0.0001
Error	17	0.08383449		

Contrast Variable: LIQUID.2

General Linear Models Procedure
Repeated Measures Analysis of Variance
Analysis of Variance of Contrast Variables

Contrast Variable: LIQUID.2

Source	DF	Type III SS	F Value	Pr > F
MEAN	1	0.24029778	12.37	0.0026
Error	17	0.33013816		

Contrast Variable: LIQUID.3

Source	DF	Type III SS	F Value	Pr > F
MEAN	1	0.39689807	11.40	0.0036
Error	17	0.59174838		

General Linear Models Procedure
Repeated Measures Analysis of Variance
Analysis of Variance of Contrast Variables

LIQUID.N represents the contrast between the
nth level of LIQUID and the mean of subsequent levels

Contrast Variable: LIQUID.4

Source	DF	Type III SS	F Value	Pr > F
MEAN	1	0.00258401	0.94	0.3464
Error	17	0.04684516		

Contrast Variable: LIQUID.5

General Linear Models Procedure
Repeated Measures Analysis of Variance
Analysis of Variance of Contrast Variables

Contrast Variable: LIQUID.5

Source	DF	Type III SS	F Value	Pr > F
MEAN	1	0.00630190	1.13	0.3029
Error	17	0.09489722		

Contrast Variable: LIQUID.6

Source	DF	Type III SS	F Value	Pr > F
MEAN	1	0.00140450	0.37	0.5491
Error	17	0.06388475		

General Linear Models Procedure
Repeated Measures Analysis of Variance
Analysis of Variance of Contrast Variables

LIQUID.N represents the contrast between the
nth level of LIQUID and the mean of subsequent levels

Contrast Variable: LIQUID.7

Source	DF	Type III SS	F Value	Pr > F
MEAN	1	0.00627200	2.22	0.1543
Error	17	0.04795867		

Contrast Variable: LIQUID.8

General Linear Models Procedure
Repeated Measures Analysis of Variance
Analysis of Variance of Contrast Variables

Contrast Variable: LIQUID.8

Source	DF	Type III SS	F Value	Pr > F
MEAN	1	0.03200450	14.34	0.0015
Error	17	0.03795400		

Contrast Variable: LIQUID.9

Source	DF	Type III SS	F Value	Pr > F
MEAN	1	0.00192200	0.53	0.4762
Error	17	0.06155600		

VITA

Rita de Nicolo Lugogo was born on May 28, 1946, in Terlizzi, a small town in Italy. Rita came to the U.S.A. when she was 6 years old. She attended the University of Connecticut where she obtained a bachelors degree in Zoology in 1968.

Upon graduation, she entered the Peace Corps and was posted in Kenya to teach science in a secondary school. She met and married Juma Lugogo during this period. After two years of Peace Corps, she was employed by Egerton Agricultural College to teach general chemistry, organic chemistry, biochemistry, genetics and physiology. When Egerton was upgraded to university status, Rita decided to return to school to pursue post-graduate studies.

Rita attended Virginia Polytechnic Institue and State University for her Ph. D. in Human Nutrition and Foods from August, 1986 through October, 1992.