

**THE EFFECTS OF BROCCOLI ON THE  
EXCRETION OF URINARY CONJUGATES**

by

**Yuni T. Kim**

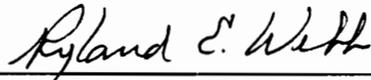
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# **The Effects of Broccoli on the Excretion of Urinary Conjugates**

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(ABSTRACT)

The effects of dietary broccoli on the body's ability to detoxify were studied in 18 male subjects between the ages of 22-40 years. The biological parameters used for measuring detoxification were the four major urinary conjugates, namely, mercapturates, sulfoconjugates, glucuronides, and amino acid conjugates. Dietary broccoli increased the urinary excretion of mercapturates and sulfoconjugates, but did not influence the excretion of glucuronides and amino acid conjugates. A significant linear trend was observed over the six-day broccoli diet treatment for both urinary mercapturate ( $P < 0.005$ ) and sulfoconjugate ( $P < 0.0001$ ) excretion. The linear trend for the mercapturate excretion was in a dose-dependent manner, resulting in a 1.3 and 2.1 fold increase by the third and sixth days, respectively, of the broccoli diet, compared to the control. For sulfoconjugates, an unexpected decrease was observed on the first day of the broccoli diet. However, within the six-day broccoli dietary treatment, a continuous increase in conjugate excretion was observed, resulting in a 2.5 fold increase by the sixth day compared to the first day. The excretion of sulfoconjugates was not necessarily dose-dependent, and increased excretion at the

highest level of broccoli (500 g) could be due to a time effect. Overall, sulfoconjugate excretion was the highest (3.98-8.91 mmole/24 h) followed by the amino acid conjugates (3.06-5.99 mmole/24 h) and glucuronides (2.85-3.54 mmole/24 h). Mercapturate excretion was the lowest (0.16-0.34 mmole/24 h). In spite of its low excretion level, the level of urinary mercapturates appeared to be the most responsive urinary conjugate to the different levels of broccoli diet.

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## INTRODUCTION

Humans are continually exposed to a variety of chemical compounds which are non-nutritive and often toxic. Some of these are bioactive endogenous compounds (e.g. steroids, prostaglandins, thyroxine, and bilirubin) indispensable to life processes, while others are non-nutritive exogenous compounds, collectively called xenobiotics, which come from a combination of environmental, occupational, dietary, social, and medicinal sources.

In primates, including humans, the endogenous compounds and xenobiotics are metabolized predominantly in the liver, intestine and to a lesser extent in the kidney or other tissue. These processes are often referred to as biotransformation. Biotransformation normally involves two stages. In the first stage (phase I), a polar functional group such as -OH, -NH<sub>2</sub>, -COOH or -SH is inserted or exposed through the process of enzymatic oxidation, reduction or hydrolysis. In the second stage (phase II), the functional group is conjugated, typically with a carbohydrate, amino acid, peptide or inorganic acid (1). The major urinary products of the biotransformation reactions in the human are speculated to be conjugates of glutathione, sulfate, glucuronic acid and amino acids (2). The conjugate has been generally considered less toxic and more water-soluble and can be readily excreted. In a strict sense, biotransformation can lead to detoxification as well as intoxication (2,3). Several

studies have shown examples of enhancement of biological activity by conjugate reactions (4-7), thus challenging the traditional concept of conjugation as a general detoxification process. However, the overall effect of such bioactivation or toxicity must be balanced against the overall detoxification metabolism since there are also various environmental factors which serve to enhance detoxification via conjugation reactions. Therefore, based on the known pathways of xenobiotic metabolism, as reflected by phase I and phase II enzymatic reactions, it would appear feasible that a conjugate excretion profile of an individual could be developed by measuring urinary metabolites which reflect the capacity to metabolize xenobiotics.

There is considerable variability among individuals in their responsiveness to xenobiotics. Furthermore, there are differences in the responsiveness of a individual given a drug on several occasions. Such inter- and intraindividual variability in xenobiotic metabolism can result from genetic differences as well as from exposure of individuals to the external environmental factors (e.g. occupation, diet, social, medicinal sources) that stimulate or inhibit drug metabolism (8-12). Compared to genetics, some external factors can be controlled by an individual. For the human, one of the greatest external factors is diet (8). This includes nutrients in the diet as well as non-nutrient chemical substances that occur naturally in food products or are developed during food processing. Dietary constituents thus have a potential to markedly influence biotransformations in the human which will significantly influence drug responses as well as toxicity caused by various environmental pollutants.

Indeed, nutrition has been known for some time to influence the biotransformation of drugs and xenobiotics in experimental animals (13-16). Also, several lines of evidence have shown the effects of specific diets on alterations of drug metabolism in humans (8,11,12). The same mechanisms that are involved in metabolizing drugs and xenobiotics are also involved in the metabolism of chemical carcinogens. Consequently, it is not surprising to find many investigations concerning the effects of diet on biotransformation relates to cancer studies. Most of these studies, however, have dealt with the effects on specific substrates or with the inducibility of specific metabolizing enzyme activities rather than the study of detoxification as an overall mechanism. The elucidation of metabolic pathways of a specific xenobiotic, including enzyme inducibility, is important for understanding biotransformation. At the same time, studying detoxification as an overall mechanism is equally important and should not be neglected. Approaching detoxification as an overall mechanism is one way of assessing the relative significance of various conjugation pathways, and, thus, gives an overall perspective.

The purpose of this research is to determine the effects of broccoli on biotransformation in adult males by measuring the urinary end products of four conjugation pathways, namely, glutathione, sulfate, glucuronic acid and amino acids. The basis for choosing broccoli is as follows. First, numerous animal and human studies have suggested that consumption of cruciferous vegetables or their extracts protects against development of cancer and other toxicities (17-21). Moreover, there

is evidence to show that the protective effect is, at least partially, due to an induction of drug metabolism activities (8,13-16,22,23). Additionally, among all the cruciferae, broccoli is consumed in substantial quantities by Western societies (22).

The hypothesis of the study is that, if broccoli has an effect on the drug metabolism pathways, the effect will be observed as a change in the urinary conjugate profile. Because the primary interest of the current study is the excretion of urinary conjugate metabolites, xenobiotics that evade detoxification and are not excreted as conjugate compounds will not be considered. This study is a part of an overall, ongoing research project whose principal objective is to develop a detoxification profile in the human, and to determine if and how external factors, such as diet, affect the profile.

## LITERATURE REVIEW

### A. Biotransformation in Humans

Most xenobiotics are extensively metabolized in the liver, intestine or other tissues prior to excretion from the body (24-26). Such metabolism is carried out primarily by two major enzyme systems. First is the mixed function oxidase system which catalyzes a variety of oxidative reactions in the endoplasmic reticulum (phase I). The second system consists of a variety of conjugating enzymes in the cytosol or endoplasmic reticulum which form conjugates either of xenobiotics or of their oxidized metabolites (phase II)(27).

The mixed function oxidase system has a structurally broad range of substrate specificities and, thus, metabolizes a variety of drugs, endogenous compounds, carcinogens and other xenobiotics. The vast majority of work with the microsomal enzyme systems has been done with liver tissue. However, current evidence (28) indicates that these enzyme systems occur in the tissues of all the major portals of entry (gastrointestinal tract, lung, skin, placenta) which provide an initial metabolic barrier to noxious foreign chemicals. Nevertheless, phase I processes do not necessarily produce deactivated or hydrophilic metabolites. A complementary group of enzymes, called the phase II or conjugating enzymes, often further metabolize

phase I products by attaching polar endogenous molecules such as glucuronic acid, sulfate, glutathione or amino acids to form water-soluble and pharmacologically inactive products that are readily excreted in the urine or bile. The attachment of polar molecules occurs primarily with functional groups such as -OH, -COOH, -NH<sub>2</sub>, -SH groups introduced or unmasked by one of the phase I pathways. In most cases the conjugation reactions involve initially an activated high energy intermediate before xenobiotics are transferred to the accepting substrate by the transferase enzyme.

There are numerous classes of conjugation reactions recognized. Of the seven conjugation reactions listed by Caldwell (2) only four are of major concern in humans. These four include, glucuronidation, sulfation, glutathione conjugation and amino acid conjugation.

## **B. Glutathione Conjugation**

Glutathione (GSH) is a tripeptide consisting of glutamic acid, cysteine, glycine and found in virtually all living organisms. It is a nonprotein peptide since the peptide bond between glutamic acid and cysteine involves a  $\gamma$  carboxyl rather than the  $\alpha$  carboxyl group of glutamic acid. An important structural characteristic of GSH is its thiol(-SH) moiety which is intimately associated with its functional role.

Approximately 90% of the total intracellular content of non-protein thiol is estimated to be GSH (30). Its concentration ranges from 0.1-10 mM depending on the tissue. Hepatocytes have levels as high as 10-12 mM. Cellular levels of glutathione are an important determinant of xenobiotic toxicity. The depletion of liver GSH stores ( $\sim 80\%$ ) have been shown to increase the susceptibility of animals to hepatotoxicity and hepatic necrosis (31,32). Glutathione, therefore, must be resynthesized constantly to meet the demands imposed by its protective role as well as its normal turnover.

Glutathione Conjugation: Glutathione (GSH) serves a variety of physiological and metabolic functions in the mammalian system, only one of which is conjugation. GSH is also known for its protective role of scavenging hydrogen peroxide, organic peroxides, and free radicals. A discussion of this role and other functions of GSH can be found in the reviews of Meister and Beutler (29,33). The following review will focus

primarily on the detoxification role of GSH via conjugation.

The ability of GSH to metabolize xenobiotics depends largely on its reactive thiol group (-SH). The thiol group, being a strong nucleophile, readily binds with potentially harmful electrophilic xenobiotics that can lead to adverse effects upon binding to vital cellular protein or nucleic acids. The adverse effects are such as hypersensitivity, cellular necrosis, hematological disorder, carcinogenesis, mutagenesis, and teratogenesis (34,35).

Figure 1 views the sequence of events of GSH conjugation. The first reaction in GSH conjugation is mediated by glutathione-S-transferase (GSt), which catalyzes the binding of a variety of electrophiles to the -SH group of GSH. GSt belongs to a group of soluble enzymes (nonmembrane bound) that are relatively nonspecific. A great number of chemically different classes of compounds are known to conjugate with GSH (36). Table 1 is a list of compounds which can conjugate with GSH. The resulting GSH conjugate (thioether) is preferentially eliminated via the bile because of its high molecular weight and amphophilic characteristics. Since components of bile may enter the enterohepatic circulation, thioethers are not necessarily inactivated and excreted from the body per se. The route that ensures inactivation and excretion is the subsequent enzymatic reaction, i.e., the removal of glutamic acid and glycine by gamma-glutamyl transpeptidase and dipeptidase, respectively, followed by formation of an N-acetyl cysteine derivative, a mercapturic acid. The mercapturic acid is predominantly excreted in the urine due to high water solubility.

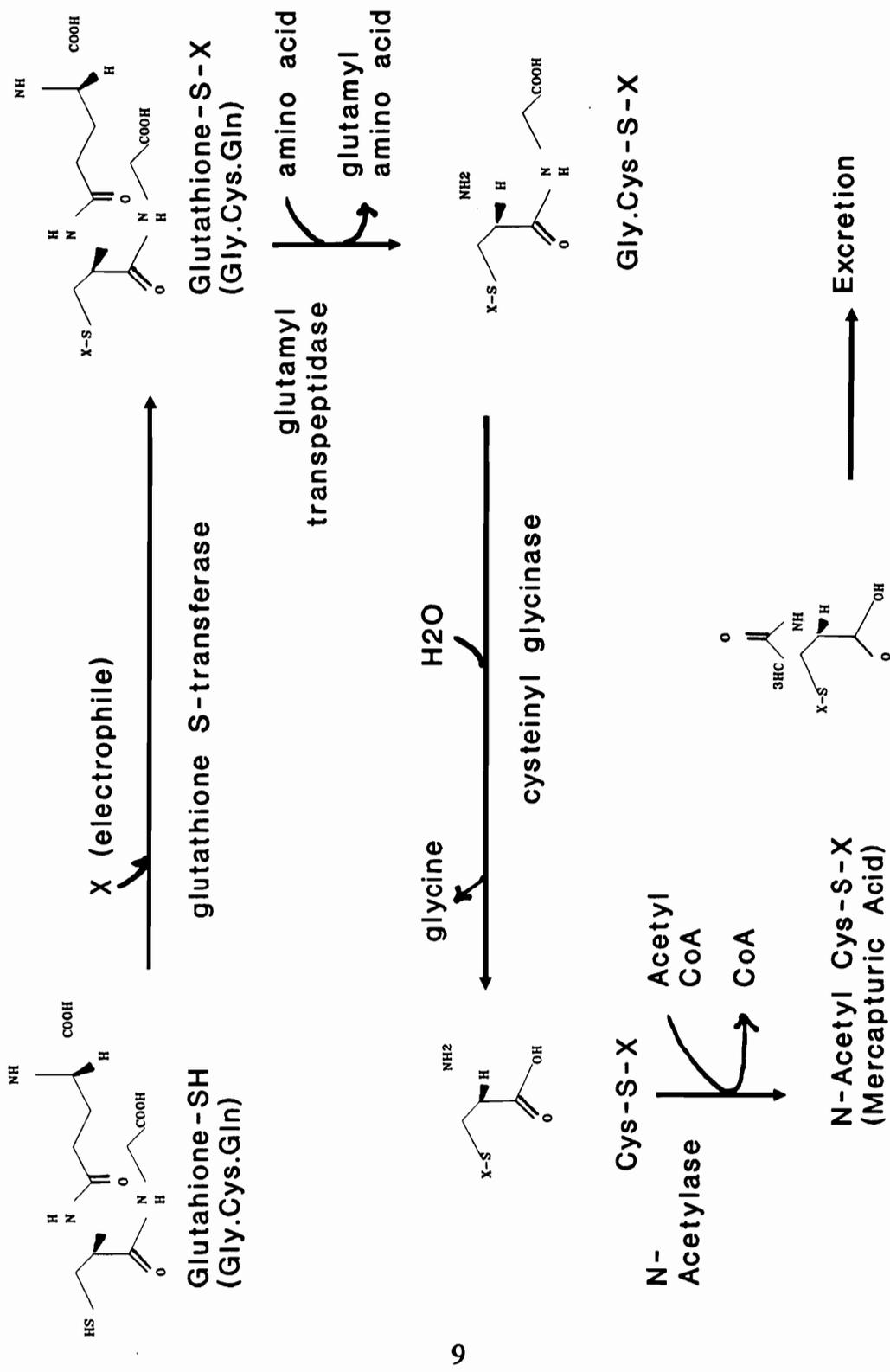


Figure 1. Glutathione conjugation

Table 1. Compounds known to conjugate with glutathione

- . Halogenonitrobenzenes and Congeners
- . 2-Chloro-S-Triazines and Congeners
- . Aryl Nitrocompounds
- . Phenol Tetrabromphthaleins
- . Aryl Alkyl Halides
- . Aralkyl Esters
- . Alkylhalides, Sulfates or Nitrocompounds
- . Alkene Halides
- . Alicyclic Halides
- . Allyl Compounds
- . Alkyl Methanesulfonates
- . Organophosphorous Compounds
- . Aryl Hydrocarbon Epoxides (areneoxides)
- . Arylhalide Epoxides
- . Other Epoxide Intermediates
  - $\beta$ -Unsaturated Compounds
- . Arylamines, Arylhydroxylamines, Carbamates, etc.
- . Steroids
- . Quinones and Catechols
- . Isothiocyanates
- . Trichloromethyl Sulfenyls
- . Thiocarbamates
- . Metals

Reproduction from Chasseaud (136)

Unlike the other three major conjugate reactions which will be discussed in the following sections, GSH conjugation does not form an initial activated coenzyme or substrate. The major prerequisite for conjugation to occur is for the substrate to be sufficiently electrophilic. Many organochemical carcinogens share the common denominator of being electrophilic themselves or being metabolically activated to electrophilic species (37). Thus, it is evident that enhancement of the glutathione-S-transferase activity or glutathione concentration could serve as a protective mechanism against cancer. Indeed, there are numerous studies on the effect of glutathione concentration and GSt activity in the development of carcinogenesis. A later section on "Influence of External Factors and Xenobiotic Metabolism" will discuss GSH conjugation in relation to carcinogenesis in more detail.

### **C. Sulfate Conjugation**

Sulfate conjugation was discovered around 1875 when Baumann found that phenyl sulfate was an important metabolite of phenol both in man and in animals (38,39). At that time, phenol was largely used as a disinfectant and antiseptic. Since then, sulfoconjugation has long been viewed as an aid to the urinary excretion of xenobiotics. Functional groups capable of forming sulfate conjugates are phenols, alcohols, arylamines and N-hydroxyl compounds (40,41). As will be discussed in the section D, many xenobiotics that can be glucuronidated can also be sulfated. There are several hypotheses as to what determines the selection between the two pathways. The limited availability of sulfate is one theory. For instance, when sulfate intake is not limiting, the ratio of sulfoconjugate to glucuronate conjugate of salicylamide and hormol increases (42-45). In addition to the sulfate level, the source of sulfur also can influence the sulfoconjugation. According to Kurzynske and Smith (42), while methionine provided adequate sulfur for sulfate conjugation, supplementing with cysteine did not have the same effect. Rather cysteine favors glucuronidation by an apparent increase in the activity of UDP-glucuronyltransferases (UDP-Gtr). Although no explanation is available, it appears that the source of sulfur may play an important role in determining the biotransformation pathway. Another factor which determines the selection between sulfoconjugation and glucuronidation is the kinetic difference between the two reactions (40,42,46). In general, sulfotransferases have

higher substrate affinity (low  $K_m$ ) than UDP-Gtr, while glucuronidation has a higher capacity (high  $V_{max}$ ). The implication is that at low xenobiotic concentration sulfate conjugation predominates and at high concentration glucuronidation predominates. The list shown in Table 2 is by no means complete, but illustrates the efficacy of sulfoconjugate and urinary excretion as a means of detoxification.

The sulfate group for conjugation comes from the pool of sulfate ( $SO_4^{2-}$ ) in the body. Serum concentrations of inorganic sulfate for man are between 0.15 to 0.5 mM (47), and reflect the balance between the dietary absorption of sulfate, the elimination of sulfate mainly by urinary excretion, and also the incorporation into sulfate conjugates.

**Conjugation Reaction:** The formation of a sulfate conjugate is, by and large, a two step process. The activation of inorganic sulfate is followed by the transfer of sulfate to the xenobiotic or endogenous substrate. Figure 2 shows the conjugation reaction as well as the structure of activated sulfate. Activation is further broken down into two steps; the formation of adenosine 5'-phosphosulfate(APS), an initial activated sulfate, and the formation of 3'-phosphoadenosine 5'-phosphosulfate(PAPS), an ultimate sulfate donor for the sulfate conjugation. The first reaction is catalyzed by ATP-sulfurylase, an enzyme which is capable of binding several moles of ATP. However, since the equilibrium of this reaction favors substrate formation, APS formation proceeds at an appreciable rate only when the products of the reaction are taken

Table 2. List of compounds eliminated as sulfoconjugates in the urine

<p>Exogenous Origin</p>	<ul style="list-style-type: none"> <li>. salicylamide</li> <li>. phenolic derivative of a thiaziazole (hypotensive drug)</li> <li>. topanol (antioxidant)</li> <li>. 5-deoxypyridoxine</li> <li>. paracetamol</li> <li>. demethylated terbutaline</li> <li>. 2-naphthylamine</li> <li>. naphthol</li> <li>. isoprenaline</li> <li>. hydroxylated metabolites of aflatoxin B</li> </ul>
<p>Endogenous Origin</p>	<ul style="list-style-type: none"> <li>. polypeptide containing L-tyrosine</li> <li>. steroids</li> <li>. serotonin</li> <li>. 2-amino-3-hydroxy acetophenone</li> <li>. 5-hydroxyindolyl acetic acid</li> <li>. dopamine</li> <li>. ascorbic acid</li> </ul>

Examples are adopted from Powell et al.(39)



away by a subsequent enzymatic reaction. Indeed, APS-kinase, which catalyzes the second reaction, has a high affinity for APS. Furthermore, the reaction is irreversible due to its high exergonic nature. Hence, the overall equilibrium of activation favors product formation, i.e., PAPS (48).

The second step of the conjugation reaction, i.e., sulfation, is the actual transfer of activated sulfate to an appropriate substrate. Transfer is mediated by a group of enzymes called sulfotransferases which are localized in the soluble cytosol fraction and found in many tissues including liver, kidney, and other tissues (41). Sulfotransferases catalyze two general types of reactions; the formation of sulfate esters ( $R.OSO_3^-$ ) and sulfamates ( $R.NHSO_3^-$ ). Both are completely ionized under physiological conditions, and thus, are very water soluble and quickly eliminated. Sulfotransferases also catalyze the formation of thiosulfates ( $R.SSO_3^-$ ); however, the reactions play no significant role in the metabolism of xenobiotics in animals (39,48).

Although the general outline of the biochemistry of sulfation is fairly clear, little information is available on sulfotransferases concerning the quantity and identity of the enzymes. It appears that a number of sulfotransferases exist, but it is not clear if sulfotransferases are a few relatively nonspecific enzymes or many highly specific ones. For xenobiotic metabolism, phenol sulfotransferase, alcohol sulfotransferase, arylamine sulfotransferase, and steroid sulfotransferase appear to be the most important (49).

Fate of Sulfoconjugates: Many difficulties arise when attempting to assess the significance of sulfation in drug metabolism. While some of the conjugates are quite clearly detoxification products of xenobiotics or inactivation products of endogenous compounds (50), others are important metabolic intermediates. For example, the sulfate moiety in connective tissues has an important functional role due to its water holding ability and cation binding affinity (51). This enables connective tissues to maintain ion exchange and balance in vivo. Furthermore, the introduction of a sulfate moiety into hydrophobic steroids, like cholesterol, serves to enable bile salts to act as surface active agents (51). The integration of sulfate into proteins is also believed to have a biological activity. L-tyrosine sulfate, a component of mammalian fibrinogen and gastrin II, is known to occur as an integral part of proteins (39,52,53). With respect to the hormonal sulfates, in some cases sulfation is a prerequisite for the hormonal effect, whereas in others sulfation is a storage form that can be enzymatically modified for activation later on (54-56).

#### **D. Glucuronic Acid Conjugation**

Glucuronidation, which was first discovered in 1855, is quantitatively considered the most common mammalian conjugation pathway (57,41). Though, it occurs in many tissues of the body, glucuronidation in the liver has received the most attention due to the high capacity of the liver for this process. Current understanding of the underlying mechanism is based on the liver glucuronidation (57,58).

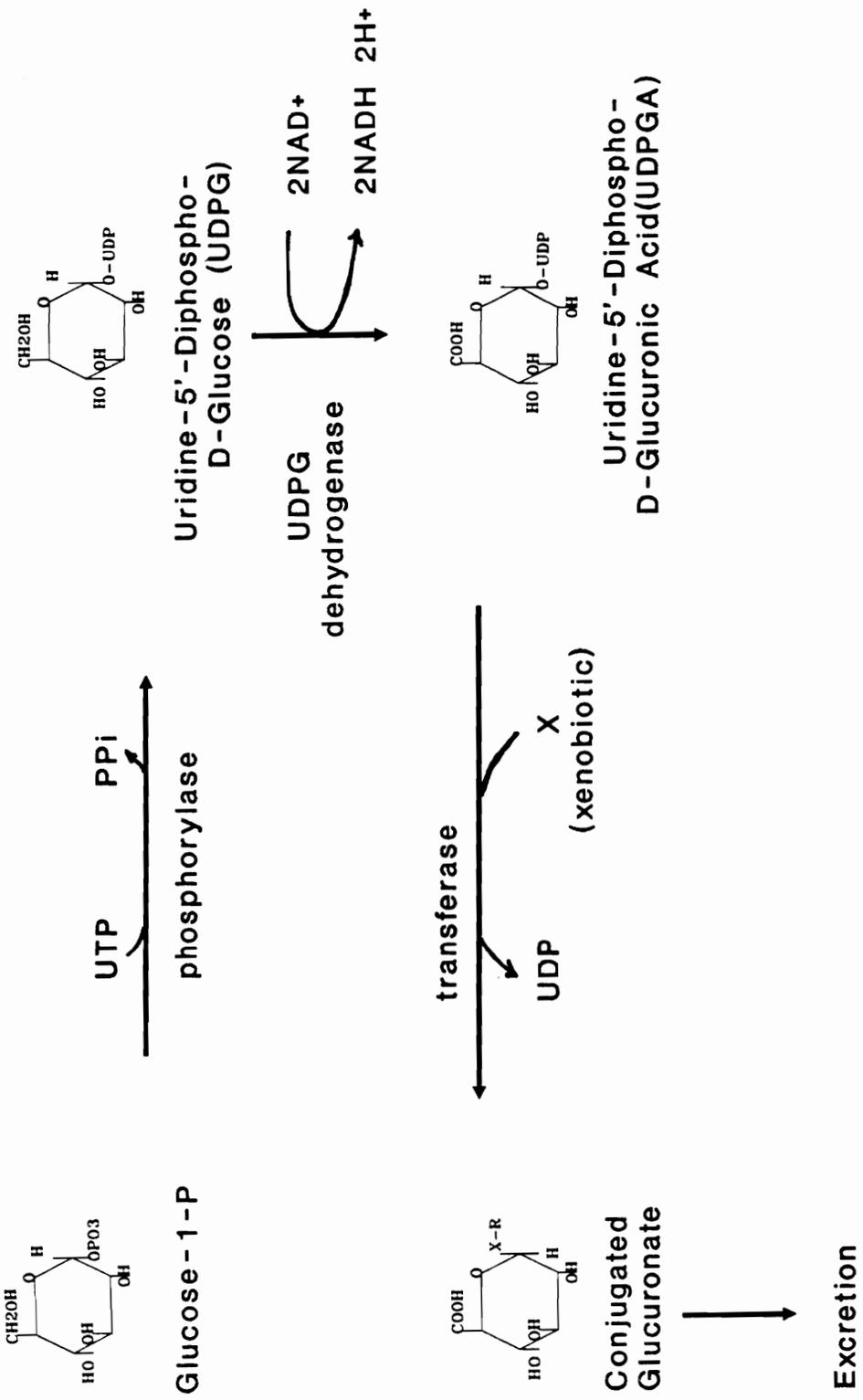
Glucuronidation, in general, involves transfer of the sugar acid moiety, i.e., glucuronic acid, to an acceptor group on the xenobiotic compound (59). A large number of xenobiotics, either exogenous or endogenous in origin, are known to be conjugated with glucuronic acid. Table 3 lists the functional groups capable of glucuronidation. There are many chemical compounds with preexisting functional groups, and also many others generate functional groups as a result of phase I metabolism. In addition to the availability of a large number of functional groups, the ubiquitous nature of UDP-glucuronic acid further increases the occurrence of glucuronidation. UDP-glucuronic acid is a product of glucose metabolism closely related to glycogen synthesis (59).

Table 3. Types of functional groups undergoing conjugation reactions with glucuronic acid

FUNCTIONAL GROUP	TYPE	EXAMPLE
Hydroxyl (-OH)	O-Glucuronide	phenols alcohols enols N-hydroxylamine
Carboxyl (-COOH)	O-Glucuronide	aryl carboxylic acids arylalkyl acids
Amine (-NH <sub>2</sub> )	N-Glucuronide	arylamines alkyl amines sulfonamides tertiary amines
Sulfhydryl (-SH)	S-Glucuronide	thiol thiophenol carbodithioic acid

Glucuronide Synthesis: Glucuronide synthesis is the reaction in which activated UDP-glucuronic acid is transferred to a substrate of endogenous or exogenous origin. As shown in Figure 3, in the conjugation reaction, an  $\alpha$ -glycosidic bond of UDP glucuronic acid turns into the  $\beta$ -configuration of glucuronides (60). This reaction is catalyzed by UDP-glucuronyl transferase (UDPGtr), a multienzyme family which comprise many forms with different substrate specificity. UDPGtrs are located in the phospholipid protein membrane, and found primarily in endoplasmic reticulum, particularly smooth endoplasmic reticulum (microsomal membrane bound) (59). Since the pioneering work on the mechanism of glucuronide synthesis done by Dutton and Smith in the early 1950s (57), there have been numerous studies on glucuronidation. However, difficulties in studying enzyme activities due to an intimate association with the membrane phospholipid layer and poor water solubility left many questions still unresolved.

There are a few incidences of glucuronidation disorder reported in humans caused by the absence of glucuronyl transferase activity or other deficiencies in the mechanism. Examples are Crigler-Najjar Syndrome and Gilbert's Disease (41,61). Additionally, neonates who do not have fully developed hepatic glucuronyl transferase activity often develop hyperbilirubinemia (physiological jaundice) and antibiotic toxicity (61).



**Figure 3. Glucuronidation**

The Fate of Glucuronides: The glucuronyl moiety, with its ionized carboxylate group and polar hydroxyl groups, gives the water solubility required for urinary excretion. Therefore, for a long time, urinary excretion was considered to be the detoxification process that terminated the parent compound (57,41,59). Glucuronides with a molecular weight of 300 or greater, however, are excreted predominantly in the bile (41). The biliary glucuronides include high molecular weight xenobiotics as well as endogenous compounds such as bilirubin and numerous steroids (35). The compounds excreted through the bile enter into the intestine where enzymatic hydrolysis may occur by  $\beta$ -glucuronidase. This results in the regeneration of free xenobiotics or endogenous compounds which re-enter the enterohepatic circulation. Therefore, conjugate excretion via the bile is not necessarily the ultimate elimination of xenobiotics per se since the conjugates may recirculate into the body.

## **E. Amino Acid Conjugation**

Amino acid conjugation was first discovered by Keller in 1842, when he showed that hippuric acid (i.e.,benzoyl glycine) found in cow's urine was formed from ingested benzoic acid (62,63). In the years following, other amino acids, such as glutamine, taurine, ornithine, and aspartic acid were also found to be utilized in conjugation reactions (63). In spite of the early discovery of these amino acid conjugates as a detoxification mechanism, they are the least investigated and understood of the four conjugation pathways. This lack of attention is probably due to the diversity of amino acids in nature and also their large functional variations. Typically, amino acid conjugation involves the carboxylic acid functional group of xenobiotics forming a peptide bond with an endogenous amino acid. Most amino acids encountered in conjugation reactions are aliphatic and dietarily nonessential in character.

Amino Acid Specificity: The specificity of an amino acid utilized in a conjugation reaction depends largely on the animal species and chemical structure of the carboxylic acid. Glutamine, for instance, is confined to the conjugation of arylacetic or heteroarylacetic acids including endogenous phenylacetic (64), indolylacetic acids (65) and only by anthropoid species (63). Taurine is relatively widespread in occurrence but conjugation is restricted to arylacetic and cholic acid derivatives.

Conversely, ornithine conjugation is versatile for the range of acids undergoing the conjugation reaction, but it is found only in some avian and reptilian species (63). Glycine, on the other hand, is the most widely spread and versatile in terms of species distribution and the type of acid that can be conjugated. Many drugs with carboxylic acid metabolites are susceptible to glycine conjugation. Salicylic acid (66,67), phenacetamide (68), isoniazid (62) the H<sub>1</sub>-histamine antagonist, brompheniramine (69), and the antipsychotic agent, haloperidol (70) are examples. Recent studies, however, indicate that the glycine conjugation, like glutamine conjugation, is more structure dependent than previously thought due to steric hindrance of carboxylic acid substrates. For instance, meta- and parasubstituted benzoic acids readily undergo conjugation with glycine, whereas orthosubstituted derivatives do not (63). Also, phenylacetic acid and its simple 4-substituted derivatives extensively conjugate with glutamine and glycine, but substitution at the alpha-carbon leads to a loss of affinity for either amino acid and conjugation occurs with glucuronic acid instead (63,71).

Free and Conjugated Amino Acids in the Urine: The amino acid elution curve obtained by Moore and Stein (72) showed 40 to 50 different ninhydrin positive substances in normal human urine. When ninhydrin positive substances were hydrolyzed and chromatographed, there was a marked increase in glycine and glutamic acid (73). Based on structure and species specificity of amino acid utilization, taurine was also considered as an important substrate for conjugation; nevertheless, the study

showed little or no increase in taurine after hydrolysis. Thus far, glycine and glutamine seem to be quantitatively the most significant amino acids excreted as urinary conjugates. Indeed, the primary conjugated amino acids excreted by normal adults are reported to be hippuric acid and phenylacetylglutamine (74,75). These compounds represent a detoxification product of benzoic acid and phenylacetic acid, by glycine and glutamine, respectively. Hippuric acid accounts for 65-75% of the observed conjugate glycine in a 24hr urine, whereas phenylacetylglutamine accounts for 50% of the conjugated glutamine (73,76).

Amino Acid Conjugation: Our understanding of the amino acid conjugation mechanism is based largely on the study of hippuric acid and phenylacetylglutamine (77-79). Amino acid conjugation is an endergonic peptide bond synthesis reaction which requires energy-rich intermediates, namely ATP and Coenzyme A. The general mechanism of reaction is summarized in Figure 4. The formation of a conjugate involves the initial activation of a xenobiotic with ATP and Coenzyme A to form an acyl coenzyme A intermediate (41,63). The activated intermediate reacts with specific amino acids (primarily glycine and glutamine in humans) to form a peptide bond. In contrast to glucuronidation and sulfoconjugation, synthesis of amino acid conjugates involves activation of the xenobiotic rather than the endogenous substrate (77,80). This is one of the unique characteristics which differentiates amino acid conjugation from the other conjugation reactions in phase II detoxification

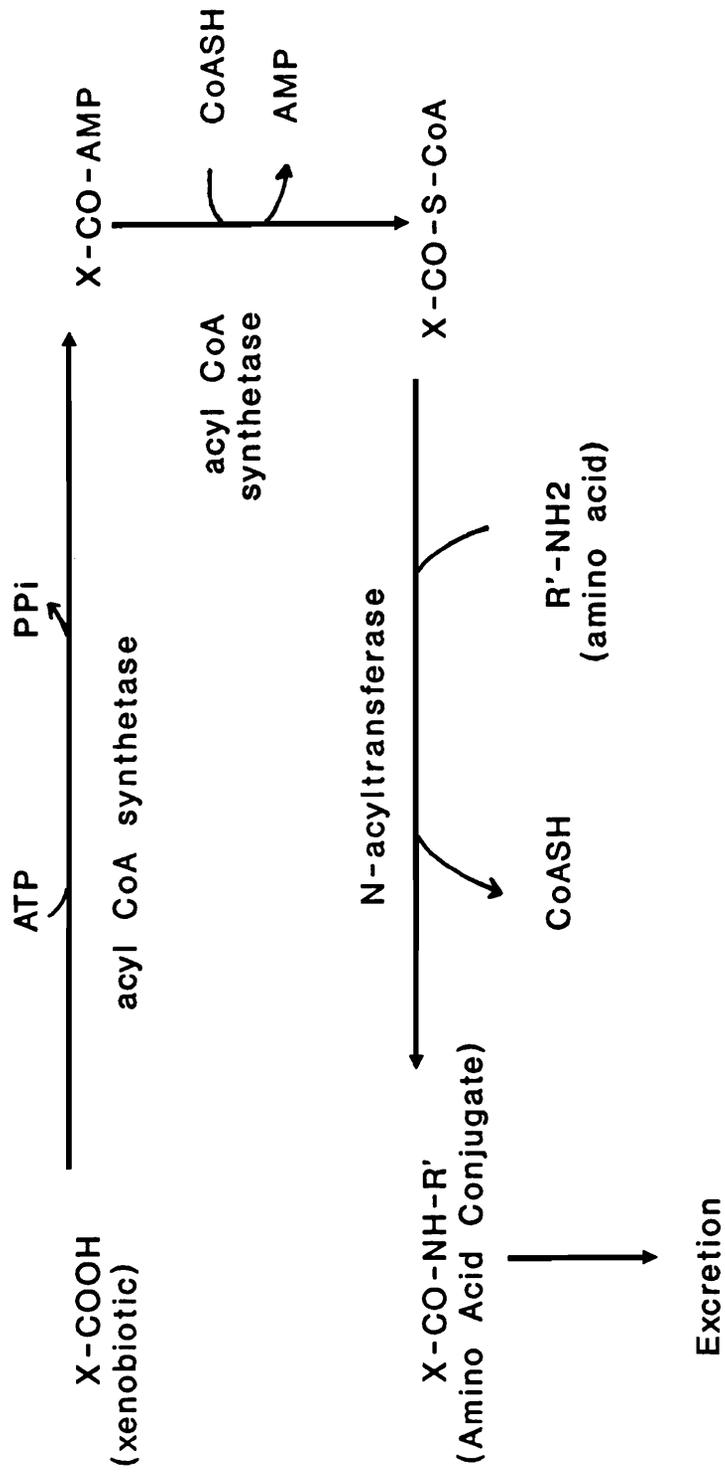


Figure 4. Amino acid conjugation

metabolism. Another distinct feature of amino acid conjugation is the subcellular location of the reaction. Whereas the other conjugation reactions are associated with the endoplasmic reticulum or cytosol, the amino acid conjugation is associated with mitochondria (63,77,81).

## **F. Influence of External Factors on Xenobiotic Metabolism**

There are a number of studies to show that glucuronic acid, amino acid, and glutathione conjugation activities are inducible by many environmental chemicals. Examples of these chemicals are insecticides, herbicides, medication, food antioxidants and naturally occurring nutritive/non-nutritive components in food (12,30,82). The mode of action is believed to be an increase in the activity of enzymes and the concentration of co-substrates involved in conjugation reactions. Studies have shown that most of the chemicals capable of inducing these enzymes also protect tissue against toxic chemicals and carcinogens (11,17,23,83-86). Therefore, it is believed that the protective roles of these compounds are at least partly due to their ability to enhance conjugation reaction of toxic chemicals and carcinogens.

### Inter- and intraindividual variation

There is considerable variability among different individuals in their capacity to metabolize xenobiotics. Furthermore, even within a single individual there is a variation from day to day. Previously in our laboratory, the daily urinary excretion levels of the four major conjugates were determined in healthy, free living, adult males (87-90). For all four pathways large intra- and interindividual variations were observed (Table 4). This large variability probably masked any significant effects of diet and environmental factors, and no strong correlation between conjugate

Table 4. Inter- and intrasubject variation in urinary excretion of conjugates in adult males

Conjugates	Mean $\pm$ SD mmole / 24 hr	Inter- variation % *	Intra- variation %
Glucuronate	0.88 $\pm$ 0.41	47	25
Sulfate	7.71 $\pm$ 5.34	69	57
Mercapturate	0.28 $\pm$ 0.08	38	32
Amino Acid	5.16 $\pm$ 0.43	52	34

\* Coefficient of Variation

Adopted from Murano, Robichaud, Kim, and Chen (87-90)

excretion and observed independent variables was noticed.

There is evidence to suggest that such inter- and intraindividual variability is partly a result of the genetic make-up among individuals. A study of twins (91,92), which traditionally is used in elucidating the relative contributions of nature and nurture, demonstrated that large interindividual pharmacokinetic variations were observed within fraternal twins but less within identical twins. Furthermore, the studies on expression of detoxification enzymes showed that a large genetic polymorphism exists among different individuals (93-95). In fact, an individual's ability to tolerate toxic or carcinogenic agents correlates with the expression of certain isoenzymes of glutathione-S-transferase (96,97).

In addition to a genetic influence, drug metabolism is further stimulated or inhibited by numerous external factors (98), which can alter the genetically predetermined biotransformation capacity. Detoxification capabilities are very dynamic, even within the same subject, and vary with time and condition. The concept of dynamic interaction and interdependence among the multiple factors was established by Vesell (98), and is represented by Figure 5. The concentric circles in the figure emphasize the infinite possible interrelationships among the external factors and the modulation by genetic factors. The multiple external factors exert such profound alterations on the gene translation and transcription mechanisms that the effects of genetics and those of external factor-induced modulation are often indistinguishable (98,99). For the human, one of the most intimate and important

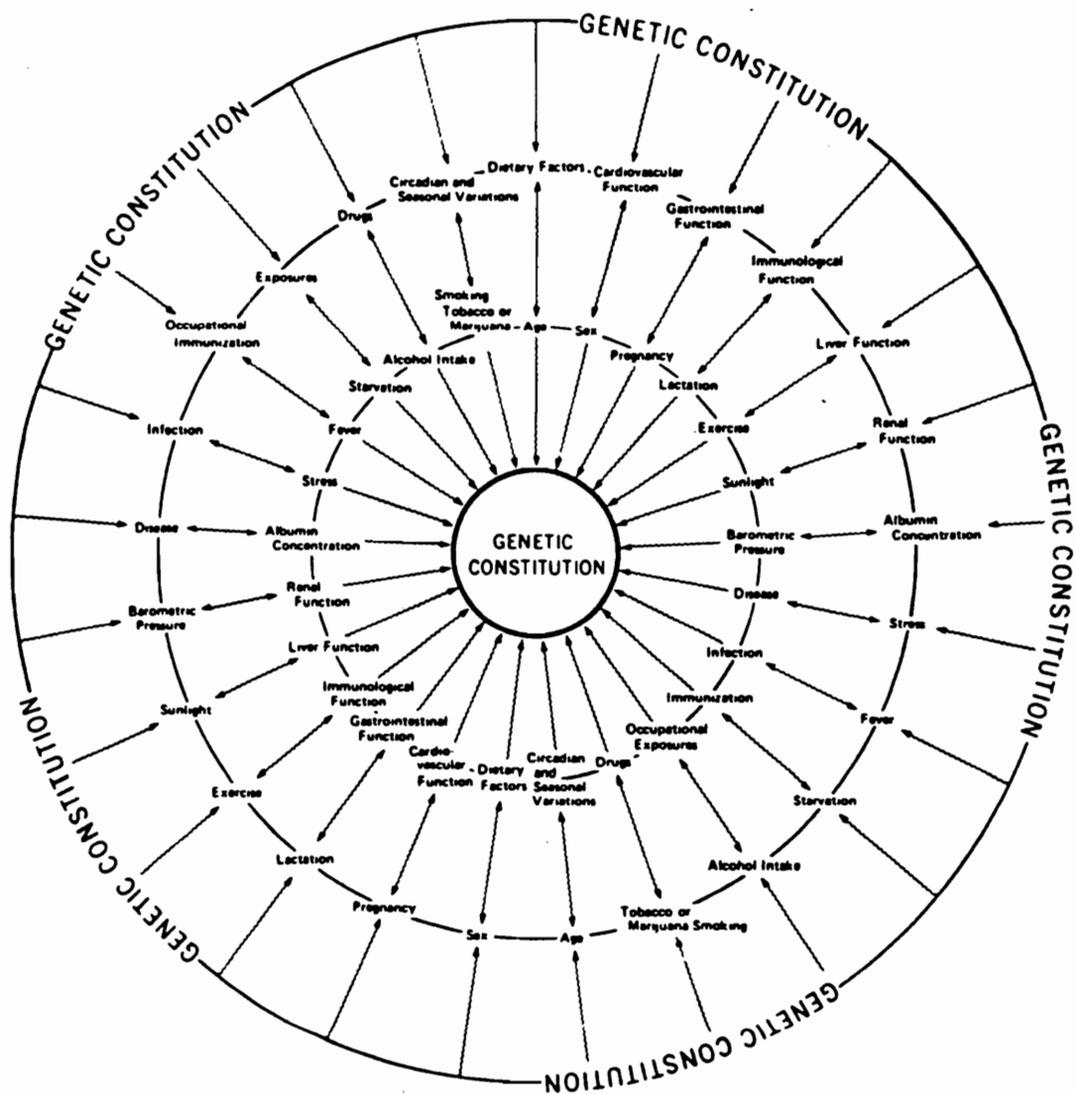


Figure 5. The complex and dynamic interrelationships between variability factors. Reproduced from Vesell (98)

external factors comes from the diet. Included are nutrients in the diet as well as non-nutrient chemical substances that occur naturally in a food or which develop during food processing. Table 5 summarizes the dietary factors that are known to influence xenobiotic metabolism.

There have been a number of epidemiological reports suggesting that a frequent intake of vegetables, especially, those of the family Cruciferae (mustards) and the genus Brassica (broccoli, cauliflower, mustard, brussels sprout, cabbage) reduce the incidence of cancer (103-105). Since these vegetables contribute a variety of ingredients to our diet, it is difficult to isolate the active component(s) that is(are) responsible for the effect on the incidence of cancer. It is quite probable that this effect is due to the multiplicity of the ingredients and not to a single agent. The evidence presented in the following section indicates that the effects of some cruciferous vegetables are due to their ability to modify xenobiotic metabolism, including various carcinogens.

#### Effects of cruciferous vegetables on carcinogen/drug metabolism

There have been a number of studies investigating the effects of natural cruciferous vegetables or their selected chemicals on xenobiotic metabolism. For example, increased intestinal *in vitro* metabolism of drugs (i.e., hexobarbital, phenacetin, 7-ethoxy coumarin and benzo-(a)-pyrene) was observed in rats fed dried

Table 5. Dietary factors that influence xenobiotic metabolism.

TYPES	EXAMPLES
preformed exogenous carcinogens	aflatoxin pyrolysates
carcinogenic precursors	nitrates N-nitrosocompounds
type and relative portion of nutrient	ratio of saturated and unsaturated fatty acids  trace elements  caloric intake
non-nutrients	fiber  glucosinolates: indoles iso- thiocyanate thione  flavone  antioxidants  phenolic compounds

Examples are adopted from references 100-102

brussels sprouts or cabbage (106). Treatment with indole compounds present in brussels sprouts and cabbage also stimulated intestinal drug metabolism, but the effect was smaller compared to the dried vegetables. Studies with human subjects also demonstrated an alteration of drug metabolism in people fed cruciferous vegetables (brussels sprouts, cabbage) (8). The mean metabolic clearance rate of antipyrine increased, with a concurrent decrease of the plasma half-life (13%), indicating a stimulatory effect of the test diet on antipyrine metabolism. Additionally, the mean plasma concentration of phenacetin decreased (34-67% at 0.5-7 h time interval), while the mean ratio of plasma conjugated N-acetyl-p-aminophenol (APAP) to unconjugated APAP increased. These results suggest the enhanced metabolism of phenacetin metabolism via increased conjugation of APAP, a major metabolite of phenacetin. Later investigations by Pantuck et al. (17) also supported the observed effects of diet on human acetaminophen metabolism. The cruciferous diet not only stimulated metabolism of acetaminophen as evidenced by enhanced glucuronidation, but also decreased production of toxic intermediates from the oxidation of acetaminophen.

There have been a series of other studies that have used carcinogens as a source of xenobiotics to evaluate the effects of cruciferous vegetables on hepatic and intestinal biotransformation. Rats fed 10-25% broccoli, cauliflower, or brussels sprouts showed a significant increase in monooxygenase activity along with the induction of glutathione-S-transferase activity for metabolism of carcinogen benzo-a-

pyrene(BaP) (13,107,108). Furthermore, the cruciferae, either in the form of intact vegetable or isolated extracts (indoles), substantially reduced (60-90% reduction) the binding of a carcinogen (aflatoxin B<sub>1</sub>) to hepatic macromolecules, i.e., DNA and protein (14,109). This was substantiated by results from other studies which showed a substantial decrease in the incidence of tumor development in rat tissues (19,110-112).

The data, thus, suggest that the consumption of cruciferous vegetables has a protective effect against toxic chemicals and possibly carcinogens. One of the postulated mechanisms for the apparent protective effects is an alteration in enzyme activity which diverts the metabolic conversion from pathways of activation to those leading detoxified products. In addition, increased reactions of active xenobiotics with noncritical cellular macromolecules such as glutathione may further enhance protection against xenobiotics.

#### Glucosinolates in cruciferous vegetables

The active components in the cruciferous vegetables that have a protective effect are believed to be certain organic sulfur compounds, most of which are derived from a common parent structure called a glucosinolate (113). Figure 6 shows the general structure of a glucosinolate and major breakdown products, i.e., thiocyanates, isothiocyanates, nitriles, and thiones. The formation of these breakdown metabolites depends on the presence of compounds which modify the action of enzymes, and

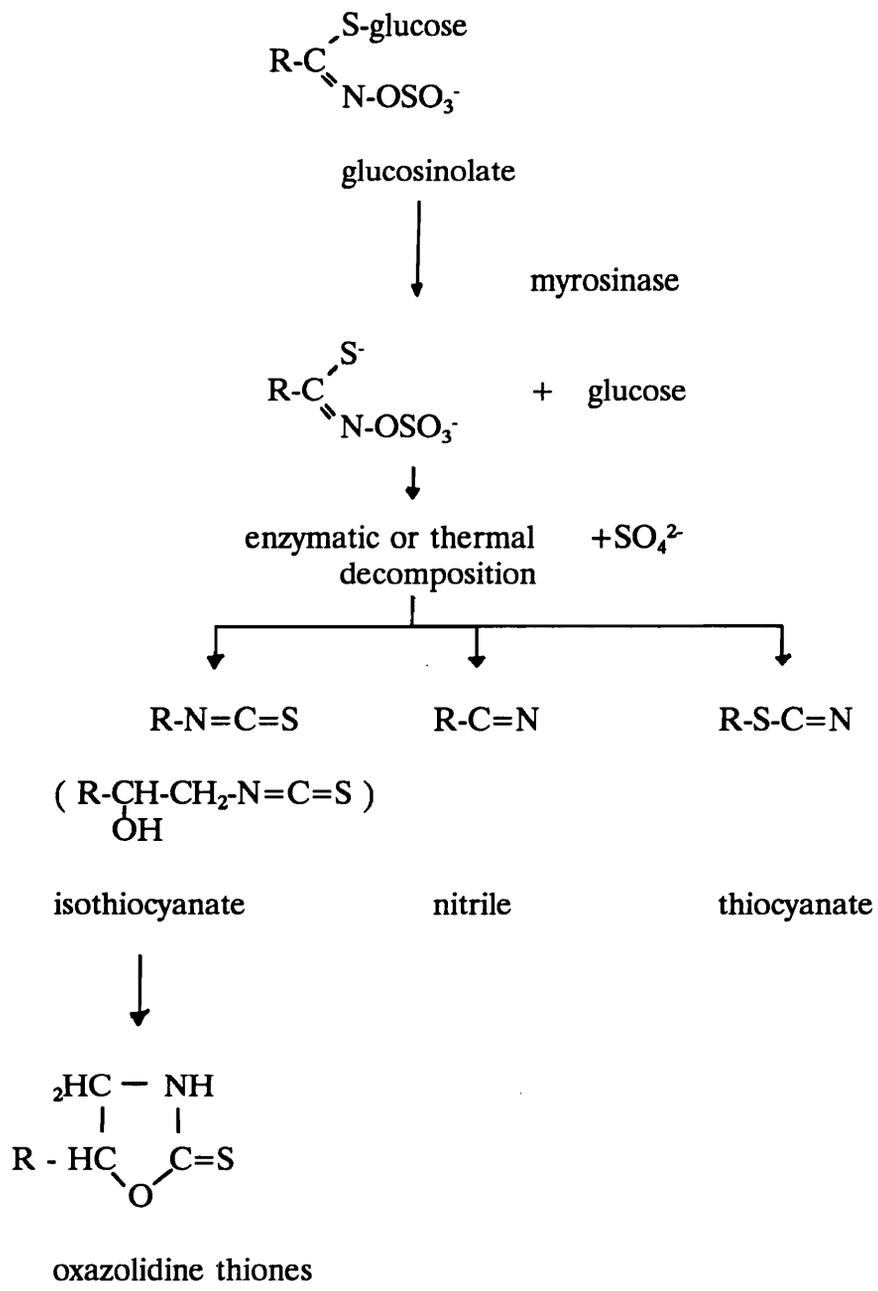


Figure 6. Glucosinolate metabolites  
(Adapted from 113, 114)

also, the aglycone (R) group of the glucosinolate. For example, under neutral conditions, glucosinolates give rise to an isothiocyanate, whereas under weakly acidic conditions or in the presence of ferrous ion, nitrile is formed. Isothiocyanates possessing a  $\beta$ -hydroxyl spontaneously cyclize to form substituted oxazolidine-2-thiones (113,114). Also the glucosinolate with indole substituted at its aglycone results in indole derivatives, i.e., indole-3-carbinol, indole-3-carboxylic acid, diindolmethane, and indole-3-acetonitrile (113,114), all of which are examples of active constituents used in the study of cruciferae extracts (Figure 7) (14,109,115-117). A further detailed discussion on glucosinolate synthesis and degradation can be found elsewhere (113,114). Based on the study conducted in Canada (118), 7.90 mg glucosinolate metabolites was consumed per person per day from 14 different brassica vegetables. A breakdown of the 7.90 mg into the three main classes showed isothiocyanate, 2.93 mg; thiocyanate, 3.56 mg; and oxazolidinethione, 1.41 mg. Thiocyanate and isothiocyanate comprised the largest percentages, 45% and 37%, respectively. Cruciferae such as cauliflower, broccoli, cabbage, and brussels sprouts were the major contributors to thiocyanates (118,119).

Recently isothiocyanato-methylsulfinyl butane, also known as sulforaphane, was isolated from broccoli (22). This isothiocyanate derivative is believed to be the key chemical in broccoli that has the most potent inducing activity for phase II enzymes. The presence of this compound in other brassica has yet to be shown; however a selective increase of phase II enzymes observed using other brassica extracts (120)

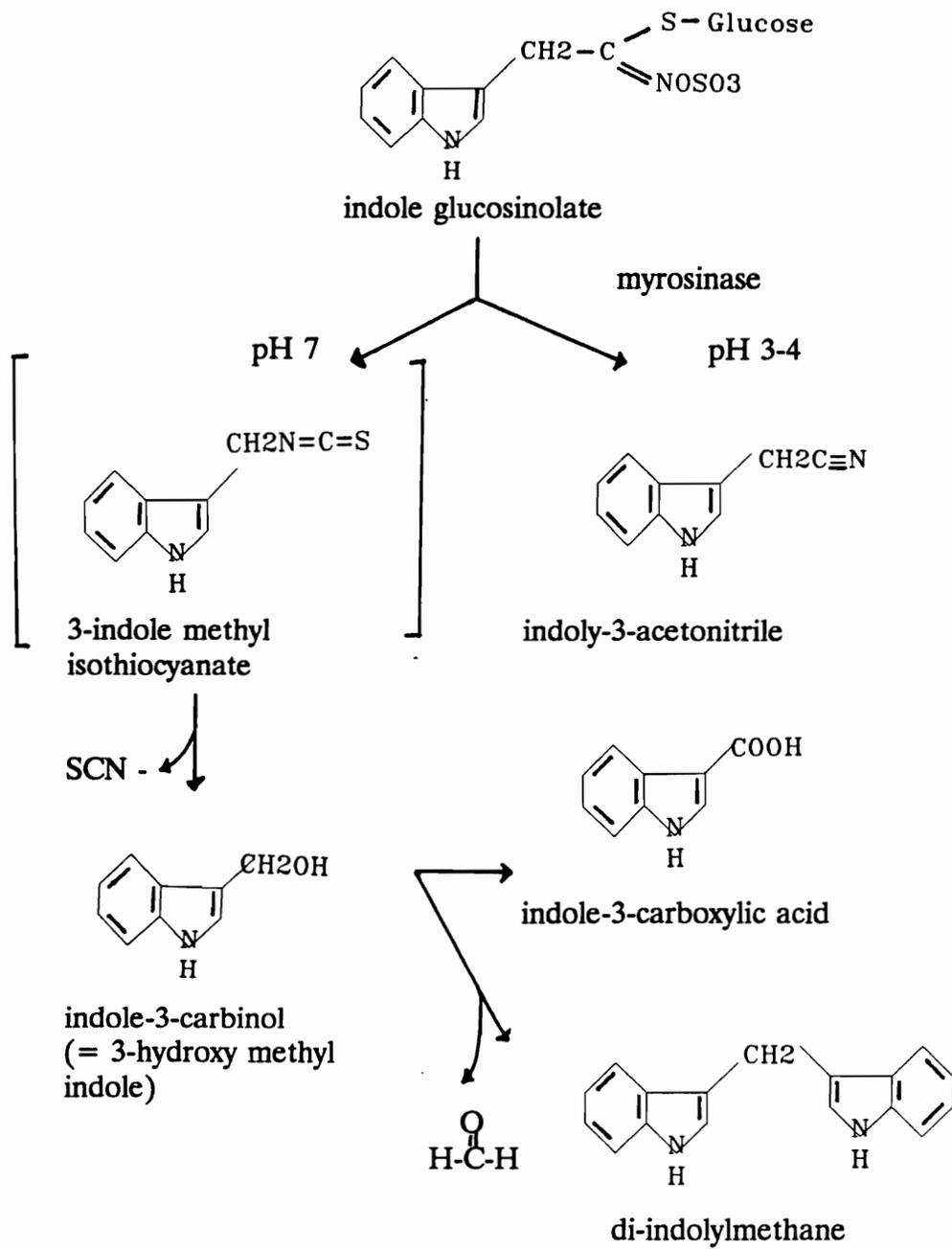


Figure 7. Indole glucosinolate metabolites

suggests an identical or at least similar compound being responsible for the effect. Discovery of sulforaphane is not entirely new however. In the past, other structurally similar isothiocyanates, such as benzyl thiocyanate, phenethyl isothiocyanate, and phenyl isothiocyanate have been investigated for their protective effect against xenobiotics and carcinogens (21,15,121-123). Like many indole derivatives, isothiocyanate also showed a protective characteristic. However, unlike many indoles, sulforaphane and benzyl thiocyanate selectively induce phase II enzymes without inducing phase I enzymes. This is a point worthy of notice since the absence of phase I induction indicates less chance of elevated cytochromes P-450 activities which could activate xenobiotics (22).

## RATIONALE

Recent recognition of the diversity and widespread occurrence of protective components in the cruciferous vegetables has led us to investigate how these vegetables, especially broccoli, may affect overall human xenobiotic metabolism.

Previously, several methods have been used to measure the xenobiotic metabolizing capacity of the human. The usual approach has been to determine the metabolites of a specific exogenous or endogenous compound(s). This approach is logical, for instance, if the assessment of the ability to control the drug dosage or the toxicological testing were necessary for product development. However, some of the methodologies are often invasive and furthermore, the test results from a specific compound may not always correlate well with metabolism of other substrates.

Within the simplistic view of biotransformation in a two step process, phase I and phase II, often it is the products of phase II conjugation reactions excreted in the urine that reflect overall xenobiotic metabolism. It is because the conjugates are usually more water-soluble than their parent compounds or phase I metabolites that they are readily excreted from the body. This view of our current knowledge of xenobiotic metabolism suggests that a profile of the xenobiotic metabolism of the human can be developed by measuring the levels of urinary end products such as conjugates. Previously, some attempts (42,44) were made to determine xenobiotic metabolism by measuring the ratio of urinary conjugates, glucuronides and sulfates.

However, the capacity of the human to withstand various xenobiotics cannot be investigated solely in terms of glucuronidation and sulfation. Glutathione and amino acid conjugation reactions, as well as glucuronidation and sulfation, are a part of the body's detoxification system, and one pathway may increase or decrease depending on the other pathway's capacity or saturation. Therefore, all four conjugates, namely, mercapturic acids, glucuronides, sulfoconjugates, and amino acid conjugates, need to be examined in order to determine one's overall xenobiotic metabolism. In the present study the levels of four major urinary conjugates were used as parameters to determine the effects of dietary broccoli on the rate of detoxification.

There are considerable advantages in employing the level of urinary conjugates as a biological parameter. First, the collection of urine is relatively convenient and noninvasive, and the analysis of conjugates is also fairly simple. Thus, the measurement of urinary conjugates can be used as a preliminary screening tool in a clinical setting for determining an individual's overall drug metabolizing capability. A second advantage is that urinary excretion takes into consideration simultaneous metabolism by hepatic and extrahepatic tissues. Although it is generally accepted that the liver is the major site of biotransformation, it is also equally recognized that extrahepatic tissues do metabolize xenobiotics. The study of detoxification from the perspective of the excreted conjugates would circumvent the need to determine the separate involvements of the hepatic and extrahepatic tissues.

In order to determine an individual's ability to cope with xenobiotic exposure

under the influence of cruciferous vegetable constituents, this study was designed to determine the effect of dietary broccoli on the four major conjugate pathways and the sum of these pathways. Considering the large number of variability factors and the complex interrelationships, it is difficult to design an experiment which could assess the impact of a single variable. Nevertheless, given the logistics of scientific investigation, it is essential to control as many variables as possible and to minimize the interindividual differences in order to detect a reproducible response (98,124). The present method, although not free from pitfalls, minimizes the variables introduced by the usual dietary intake by placing subjects on a nutritionally balanced semisynthetic diet. Also to reduce the interindividual differences which would mask any existing correlation between the urinary conjugates and the external factor of interest, each individual served as his own control.

## **METHODOLOGY**

### **A. Subject Recruitment**

A total of 18 healthy adult males who were not taking medications or under a physician's care were recruited for the study. The ages of the subjects were between 22 and 40 years. Subjects were initially screened according to selection criteria (Table 6) as per the survey questionnaire (Appendix A). Volunteers who were selected and completed the study were compensated.

Prior to the urine collection, a meeting was scheduled for the purpose of explaining the objectives of the study. Additionally, the rights and responsibilities of the subject, the possible inconveniences and benefits of participation, and the detailed procedures for urine collection were carefully explained as required by the Institutional Review Board for Research with Human Subjects. All volunteers who decided to participate were asked to sign the Informed Consent Form (Appendix B). Personal information was also obtained for contacting purposes.

Table 6. Subject selection criteria

- No use of tobacco, drug, or medications
- Limited alcohol and caffeine consumption prior to the experiment ( $\leq 3$  times a day)
- No alcohol and caffeine consumption during the experiment
- No recent weight gain or loss
- No excess exercise program, e.g., athletes, weight lifters, etc.
- Age between 22-40 years
- Male caucasian

## **B. Experimental Design**

The urinary conjugates were monitored in the subjects under three different dietary regimens. Figure 8 shows the flow chart of the overall experimental design.

During the initial three days of the study, each individual's usual level of urinary conjugates was determined and was defined as Normal Diet period (ND). During this time subjects maintained their usual dietary intake and kept a food diary (Appendix C), accurately weighing or measuring all foods and beverages consumed. The mean daily caloric value of the three-day intake was analyzed using Nutritionist III program, and used as a basis for establishing the amount of semisynthetic diet in the subsequent diet treatment period which was defined as Liquid Diet (LD). The semisynthetic diet selected for use was a nutritionally balanced defined formula with fiber (Enrich from Ross Laboratories, Columbus, OH). Nutrient components of the semisynthetic diet are shown in Table 7. Based on a pilot study, it was estimated that a minimum of seven days were required to reach stable or baseline levels of urinary conjugates with the semisynthetic diet. Once a stable baseline was established, the subjects consumed a cruciferous vegetable, i.e., broccoli, in addition to the semisynthetic diet. This period was defined as Broccoli Diet (BD). BD was further subdivided into two periods; 250 g broccoli was fed per day during the first three days and 500 g broccoli was fed per day during the later three days. Broccoli was microwaved for 6-8 min on high power (2450 MHz).

n=18 (male age 22 - 40)

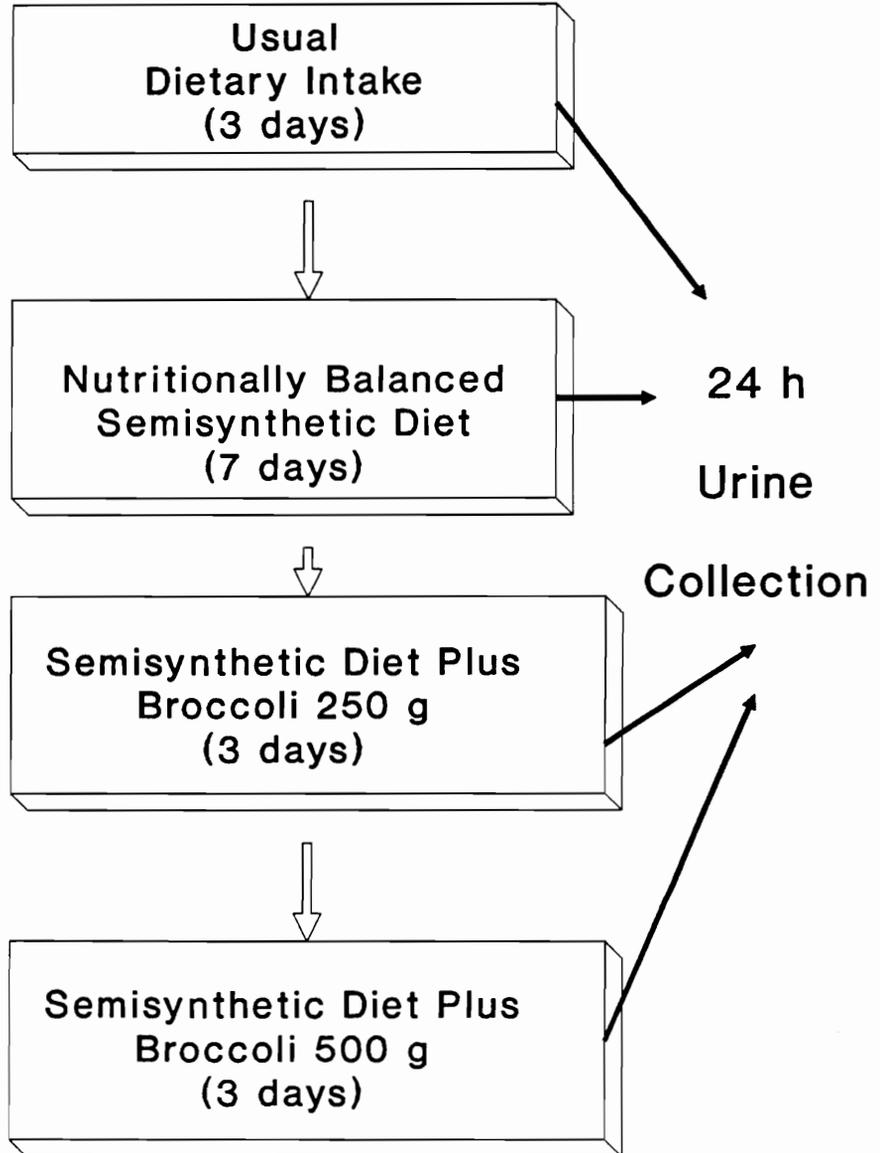


Figure 8. Experimental Design

Table 7. Nutrient components of the semisynthetic diet  
(Enrich: Ross Laboratory, Columbus, OH)

Nutrients	% Kilocalories	Grams/8oz
Protein	14.5	9.4
Sodium & 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
Carbohydrate	55.0	38.3
Hydrolyzed Cornstarch		22.2
Sucrose		12.2
Soy Polysaccharide		3.8
Dietary Fiber	-	3.4

\* 270 Kilocalories per 8 oz can

About six 8 oz can provide 100% of U.S. RDA for Vitamins & Minerals

Also, to maintain consistency, a single commercial brand of broccoli was used. Subjects consumed the required amount of broccoli all at once at the Food Preparation Lab (Dept. of Human Nutrition & Foods, VPI&SU) during their lunch hour. To reduce the interindividual differences, each subject served as his own control.

### **C. Urine Collection**

Subjects were required to collect 24 h urines throughout the entire experimental period. A 24 h period was defined as beginning with the collection of the second voiding on day one, through the first voiding on day two. Urine was collected in labeled, polyethylene, wide-mouth containers. The total volume and the pH of urine samples were determined daily on delivery. Each subject's urine sample was subsampled into several aliquots and immediately frozen for future analysis. Upon each delivery, the body weight of the subject was recorded to ensure no change of weight occur during the experiment period (Appendix D). Additionally, any deviations in the urine collection and handling procedures (e.g. incomplete collections, spillage, ingestion of medications) were promptly reported at this time. Instructions for collection and handling of urine are shown in Appendix E.

#### **D. Urinary Creatinine Determination**

The daily creatinine excretion was used for determining an appropriate dilution factor for conjugate analysis, when needed. A Creatinine Analysis Kit (No. 555) purchased from Sigma Diagnostics (St. Louis, MO) was used. The method is based upon the characteristic property possessed by creatinine which yields a red tautomer of creatinine picrate in the presence of picric acid in alkaline solution. The reaction is commonly known as Jaffe reaction. The absorbance was read at 500 nm (Milton Roy Spectronic 601).

#### **E. Conjugate Determination**

##### Mercapturic Acids:

Urinary mercapturic acids were determined by a modified method of Seutter-Berlage (125) (Figure 9). The method is based on the use of Ellman's reagent, which selectively reacts with free thiol (-SH) groups to yield thionitrobenzoate. The resulting thionitrobenzoate served as the basis for a quantitative spectrophotometric assay at 412 nm (Milton Roy Spectronic 601). To release the bound sulfur of the mercapturic acids the urine samples were hydrolyzed using 5M NaOH. The level of mercapturic acids was then calculated as the difference between alkaline hydrolyzed and

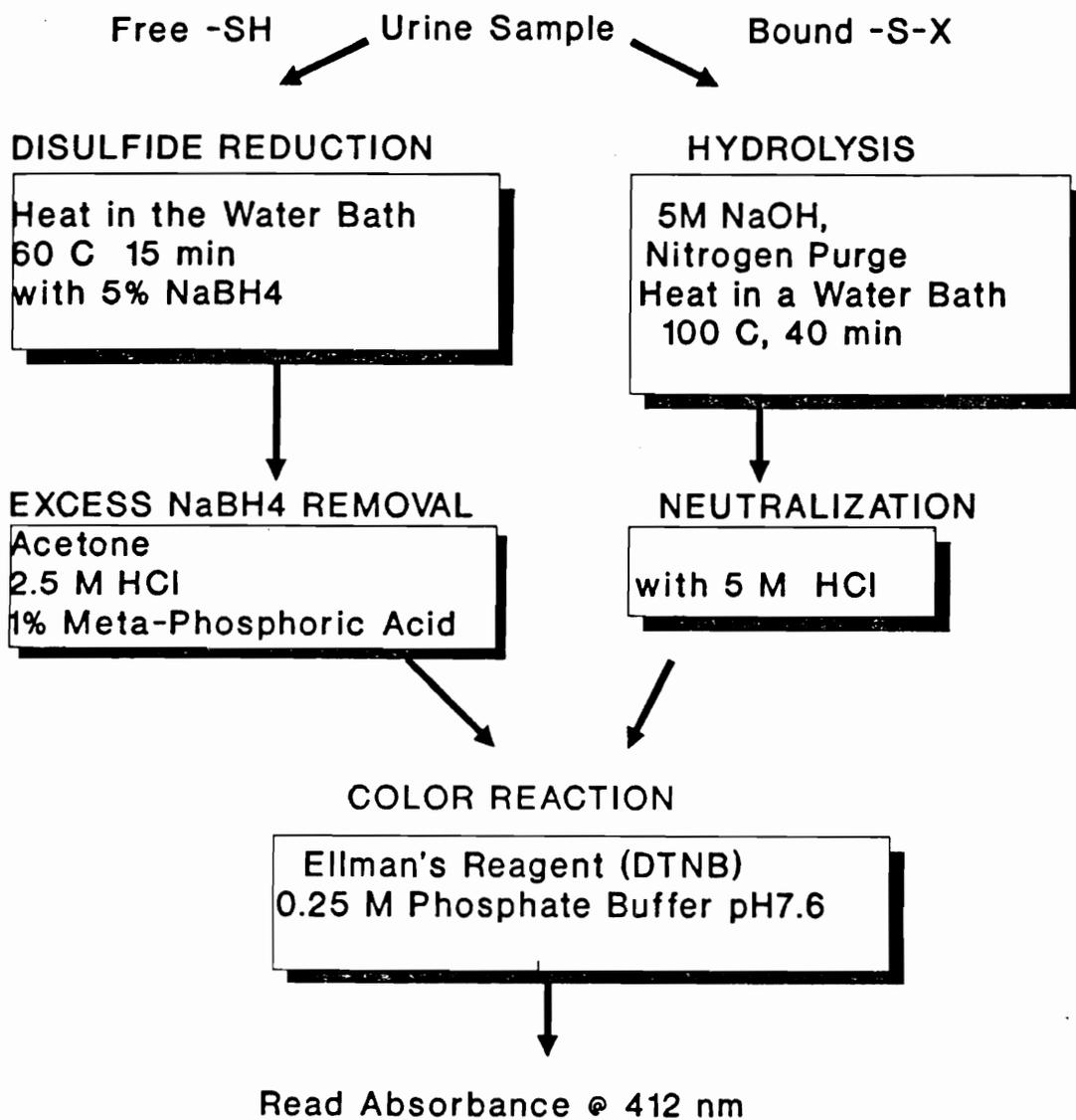


Figure 9. Determination of mercapturic acids

nonhydrolyzed urinary thiol. N-Acetyl Cysteine spiked with a urine sample was used for the standard curve development. Analyses were done in triplicate, with triplication in the range of 0-5% for the coefficient of variation. Detailed procedure is given in Appendix F.

#### Sulfate Conjugates:

Atomic absorption of barium in barium chloride-precipitated sulfate (126) was used to quantify conjugated sulfates (Figure 10). To hydrolyze conjugated sulfates, the urine sample was heated at 100° C for 24 h with fuming nitric acid. Barium chloride was added to precipitate the hydrolyzed sulfate. The resulting barium sulfate pellet was washed twice with deionized water and then barium was determined using a Model 2100 Atomic Absorption Spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). The level of conjugated sulfates was calculated as the difference between acid hydrolyzed and nonhydrolyzed urine sulfate. Sodium sulfate was used for the standard curve preparation. Analyses were done in triplicate, with triplication in the range of 0-5% for the coefficient of variation. Detailed procedure is given in Appendix G.

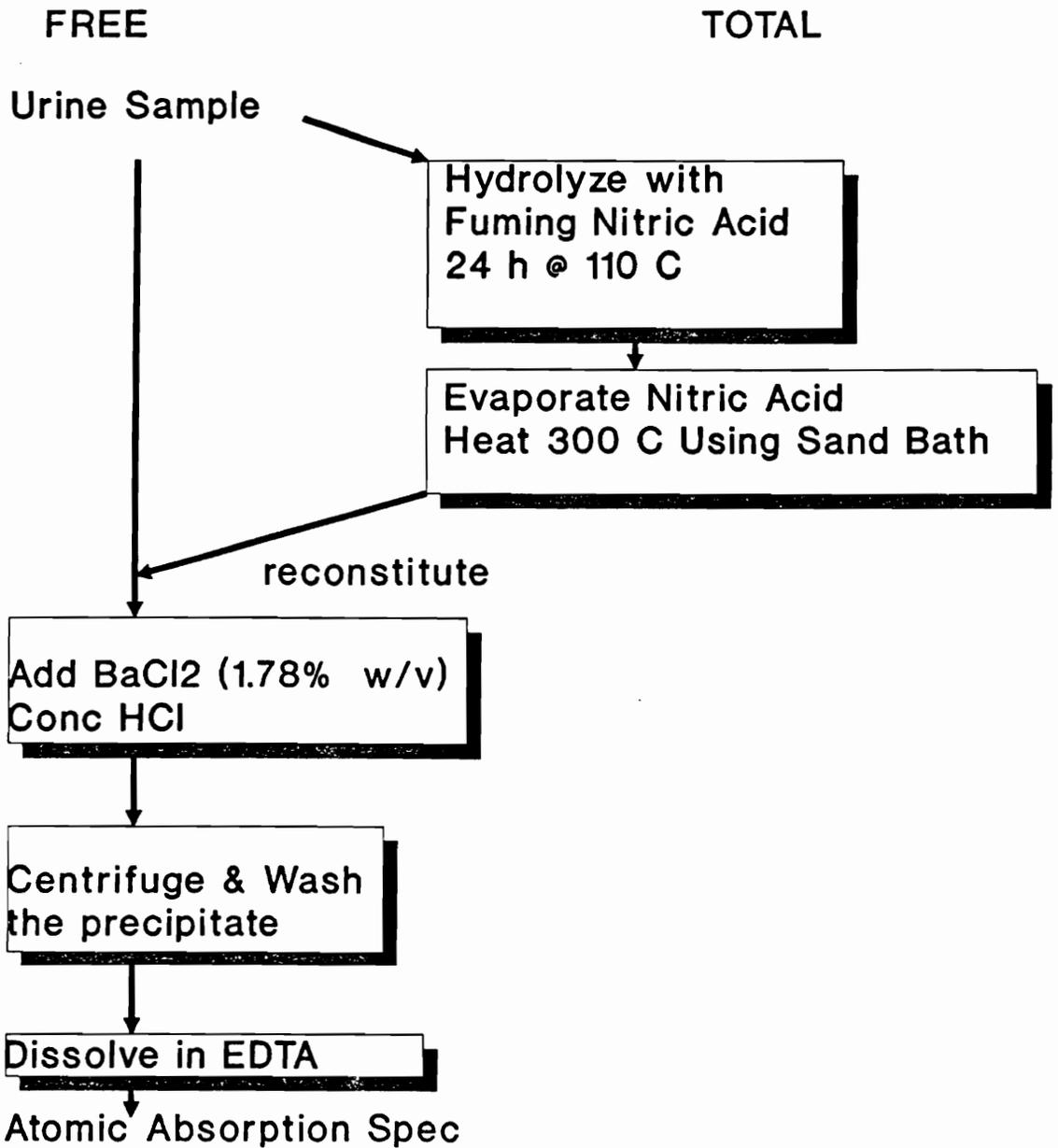


Figure 10. Determination of Sulfoconjugates

### Glucuronides:

A modified naphthoresorcinol method was used to quantify conjugated glucuronic acids (127) (Figure 11). To remove possible interfering substrate, i.e., free glucose and free glucuronic acid, the urine sample was treated with glucose oxidase and 2N NaOH prior to color development. Once the samples were subjected to the naphthoresorcinol treatment, glucuronate conjugates were extracted with ethylacetate and the absorbance was read at 564 nm on a Milton Roy Spectronic 601 spectrophotometer. Mono- $\beta$ -Glucuronic Acid was used for the standard curve development. Analyses were done in triplicate, with triplication in the range of 0-5% for the coefficient of variation. Detailed procedure is given in Appendix H.

### Amino Acid Conjugates:

The method of Bidlingmeyer et. al. (128) was used for the quantification of urinary conjugated amino acids (Figure 12). The method is based on the use of Edman reagent, which yields phenylthiocarbonyl derivatives of amino acids. The derivatives of these compounds were separated and analyzed using a PICO.TAG Waters High Performance Liquid Chromatography system. Vaporized 6N HCl was used to hydrolyze conjugated amino acids. Quantification of the conjugates was then determined by calculating the differences of each amino acid before and after conjugate hydrolysis. Eighteen subjects were randomly divided into three groups of

Urine Sample



Acetate Buffer 0.1 M, pH 5.6  
Glucose Oxidase Type V  
Incubate w/ Cont' Aeration  
(45 min @ 37 C)

GLUCOSE  
OXIDATION



2N NaOH, Boil 20 min

GLUCURONIC ACID  
DECOMPOSITION



Naphthoresorcinol Reag  
Conc HCL Boil 30 min

COLOR  
REACTION



Ethylacetate

REACTION PRODUCT  
EXTRACTION



Read Absorbance @ 564 nm

Figure 11. Determination of glucuronides

Free Amino Acids

Bound Amino Acids

Ultrafiltered  
Urine Sample



Vacuum Dry  
to 70 mTorr



6N HCl  
Vaporized using  
Slight Vacuum (1-2 Torr)



105 - 112 C Oven  
24 h



Vacuum Dry to 50 mTorr

Add Redrying Agent  
Vacuum Dry Again  
to 50 mTorr



Derivatize w/ PITC



HPLC Injection

Figure 12. Determination of amino acid conjugates

six each, and the subjects' urine samples within each group were pooled for the analyses. Samples were run in duplicate. Detailed procedure is given in Appendix I.

## **F. Data Analysis**

Repeated Measure of Analysis of Variance was performed on the excretion levels of mercapturate, sulfoconjugate, and glucuronides before and after introduction of the broccoli diet to determine the statistical differences ( $P < 0.05$  or less) between the diet treatments. Student-t-Test was used for the amino acid conjugate excretion. To determine the trend within the broccoli diet, Repeated Measure of Analysis of Variance using Contrast Variables was used. To further determine the dose response of the different levels of broccoli diet, Multivariate Analysis of Variance was used.

## RESULTS

Eighteen subjects recruited for the study successfully completed the 16 day experimental protocols. The subjects' compliance for 24 h urine collections and dietary consumptions was excellent. The mean body weight changes of 18 subjects during the 16 days experimental period was  $76.5 \pm 0.4$  Kg. Each subjects mean body weight  $\pm$  SD is shown in Appendix D. Table 8 shows the mean macronutrient and dietary fiber consumption of 18 subjects during each diet regimen. The kilocalorie distribution of each macronutrients were fairly constant throughout the experimental period. However, the dietary fiber consumption increased during the semisynthetic (or liquid) diet (LD) (55%), and more so during the broccoli diet (BD) (77-105%).

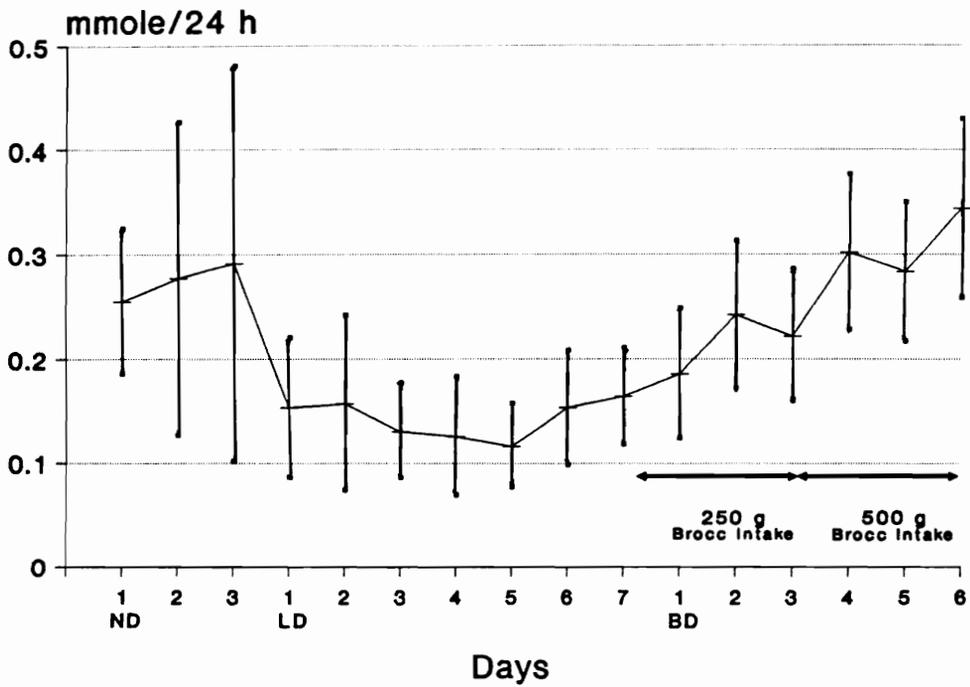
### A. Mercapturates

Figure 13 shows the changes in average mercapturate excretion over time. Although a rise was noticed at LD6 and LD7, the overall mercapturate excretion level was lowered by the liquid diet. At LD7, mercapturates were 0.16 mmole /24 h, a 40% decrease from that of the average ND excretion level (0.27 mmole/24 h). When broccoli was given in addition to the liquid diet, the excretion level increased

Table 8. The mean nutrient consumption over experimental period (n=18)

Diet	Kilocalories	% Kilocalories			Dietary Fiber (grams)
		Carbohydrate	Protein	Fat	
Normal	2811	54	15	31	22
* Liquid	2700	57	14	29	34
plus 250g Brocc	2768	57	14	29	39
plus 500g Brocc	2835	58	15	27	45

\* Enrich (10 8oz cans: Ross Laboratory, Columbus, OH)



ND = normal diet  
 LD = liquid diet  
 BD = broccoli diet

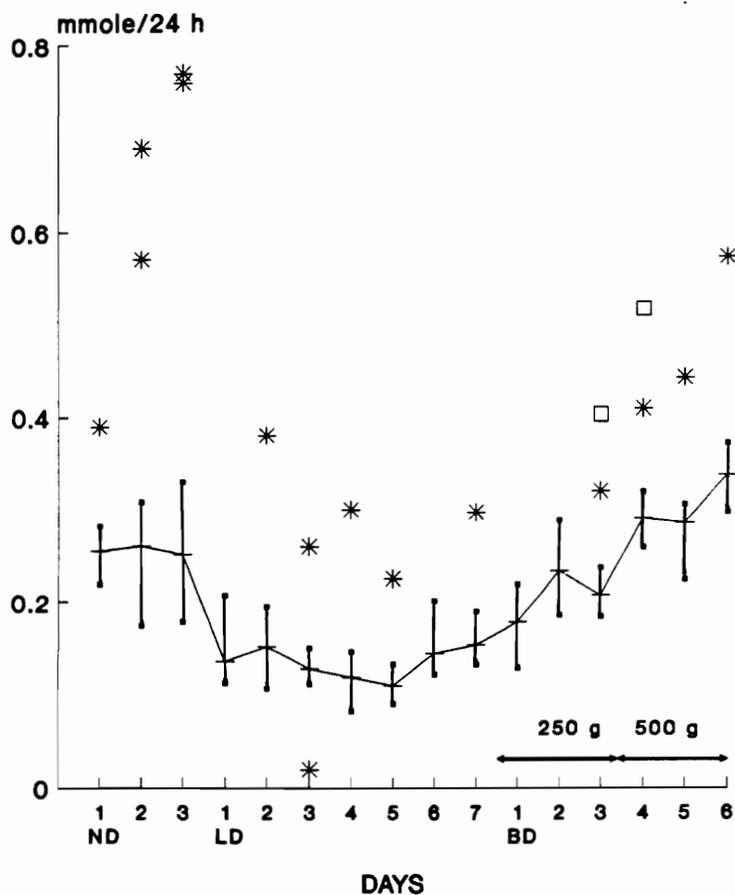
Figure 13. The mean( $\pm$ SD) urinary mercapturate excretion of adult males. (n=18)

over time resulting in a 25% increase at BD6 compared to the average excretion of ND. To examine whether the overall excretion profile was largely influenced by the outliers, the excretion level expressed in median values was also plotted (Figure 14). The median trend was similar to that of the mean, suggesting that the changes observed were not influenced by the outliers.

A significant ( $p < 0.005$ ) linear trend was observed over the six day treatment with a correlation coefficient value of 0.56 for all the 18 subjects. The correlation coefficient of the mean values was 0.86 (Figure 15). When the two levels of broccoli (BD1-BD3 250 g ; BD4-BD6 500 g) were compared (Figure 16), the mean urinary mercapturates were different ( $P < 0.0005$ ). Furthermore, an ( $P < 0.05$ ) intercept difference was observed between the two levels, indicating a jump in the mean urinary mercapturates with the increased broccoli intake. However, there was no ( $P > 0.05$ ) slope difference between the two levels, indicating that there was no change in excretion rate with increased broccoli intake.

## **B. Sulfoconjugates**

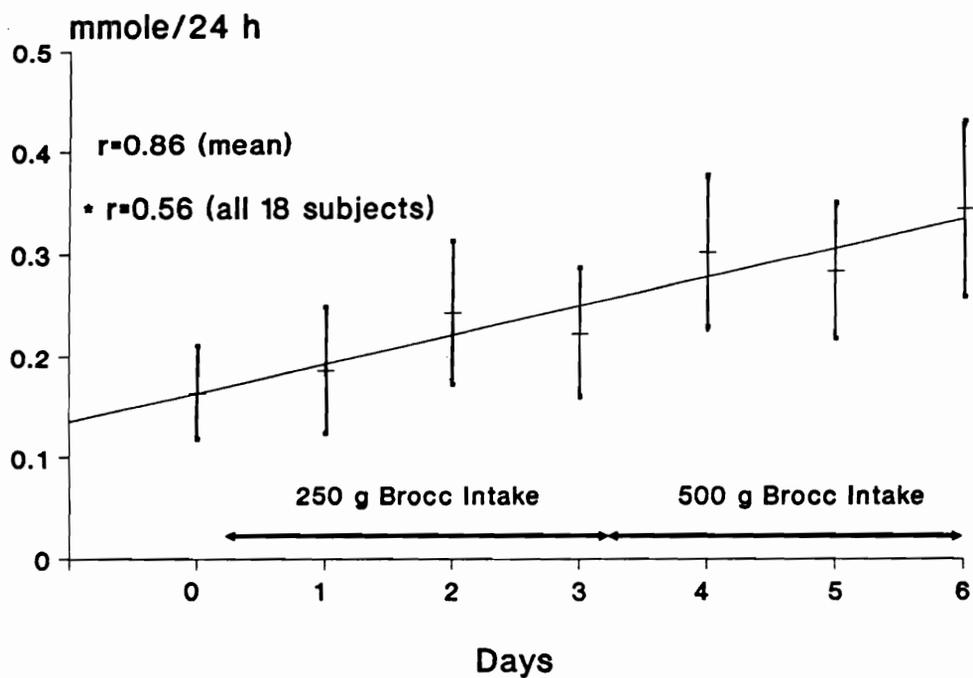
Figure 17 shows the changes in mean sulfoconjugate excretion over time. The liquid diet initially lowered the conjugate excretion resulting in 4.32 mmole/24 h at LD3, a 20% decrease compared to that of the average excretion level of ND (5.43



ND = normal diet  
 LD = liquid diet  
 BD = broccoli diet

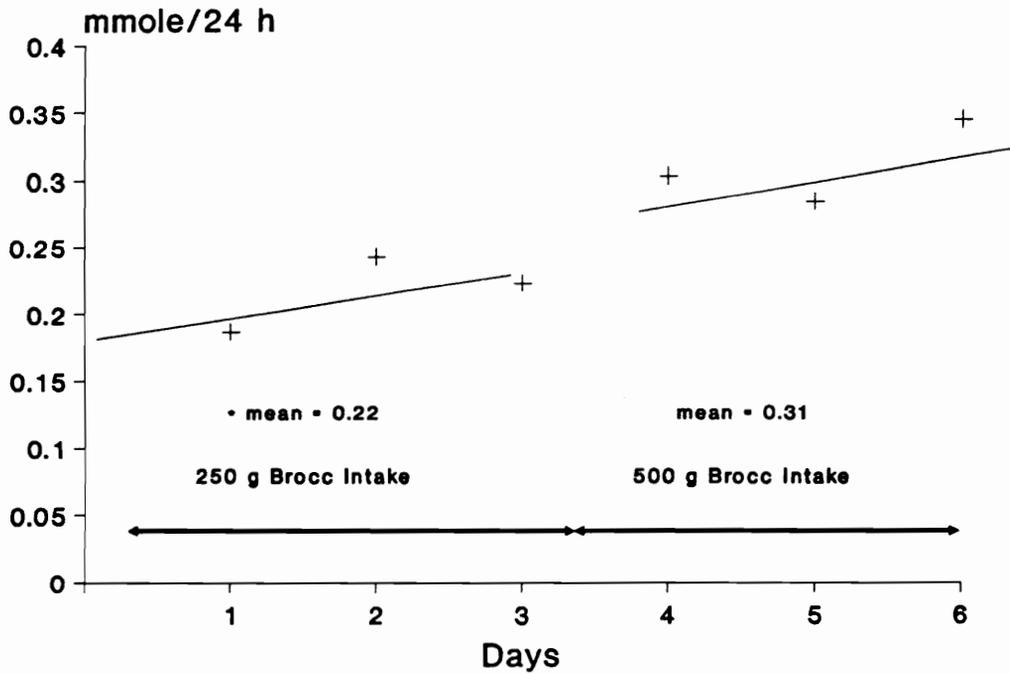
— interquartile 25-75%  
 \* at least 1.5X out of quartile  
 □ at least 2.5X out of quartile

Figure 14. The median urinary mercapturate excretion of adult males. (n=18)



\* A linear increase of excretion level was observed ( $P < 0.0005$ ).

Figure 15. The effect of dietary broccoli on mean( $\pm$ SD) urinary mercapturates in adult males.

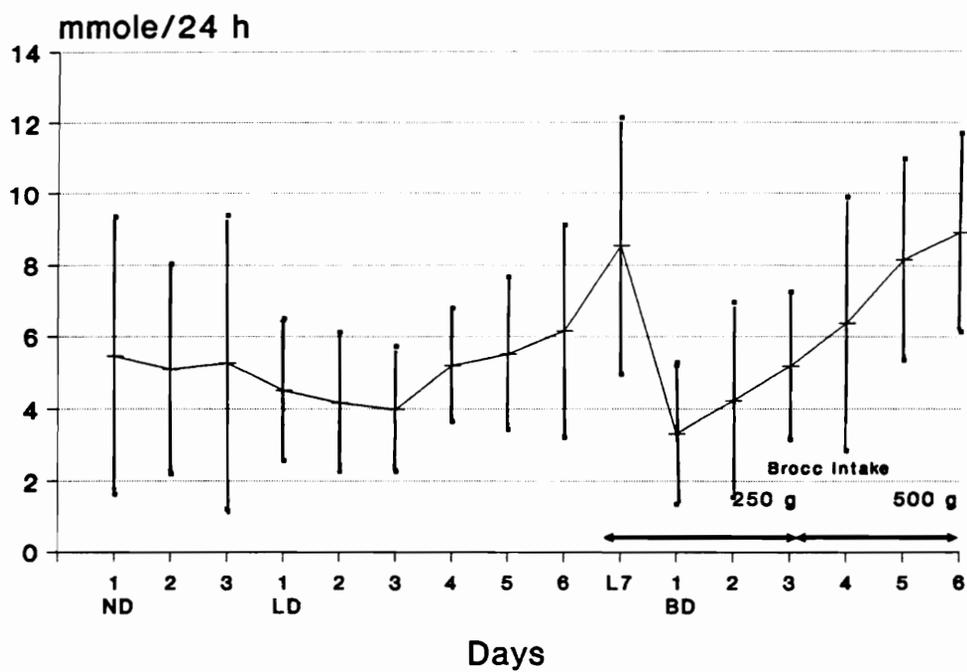


\* Mean excretion values of the two levels were different ( $P < 0.0005$ ).

Intercepts of the two levels were different ( $P < 0.05$ ).

Slopes of the two levels were not different ( $P > 0.05$ ).

Figure 16. The effects of different levels of dietary broccoli on mean urinary mercapturate in adult males.(n=18)



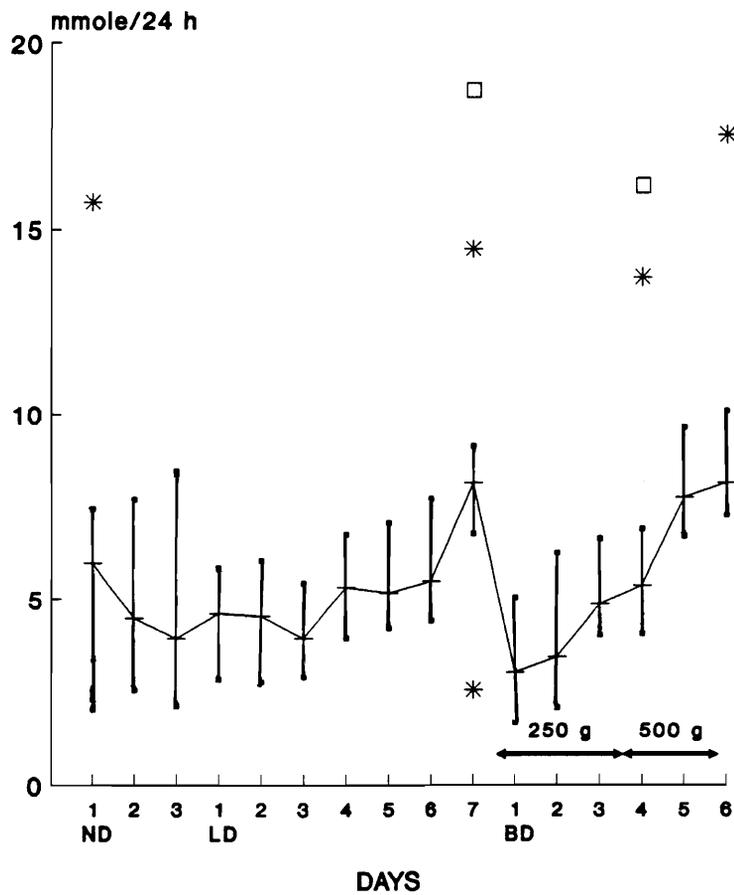
ND = normal diet  
 LD = liquid diet  
 BD = broccoli diet

Figure 17. The mean( $\pm$ SD) urinary sulfoconjugate excretion of adult males. (n=18)

mmole/24 h). However, a persistent rise was noticed thereafter, reaching a 54% increase at LD7 (8.36 mmole/24 h) compared to that of the average excretion level of ND. At BD1, the first day of the broccoli diet, a drastic decrease (57%), in conjugate excretion was observed relative to LD7. Figure 18 is the excretion profile expressed in median values. The median trend was similar to that of the mean, suggesting that the apparent changes observed were not influenced by the outliers.

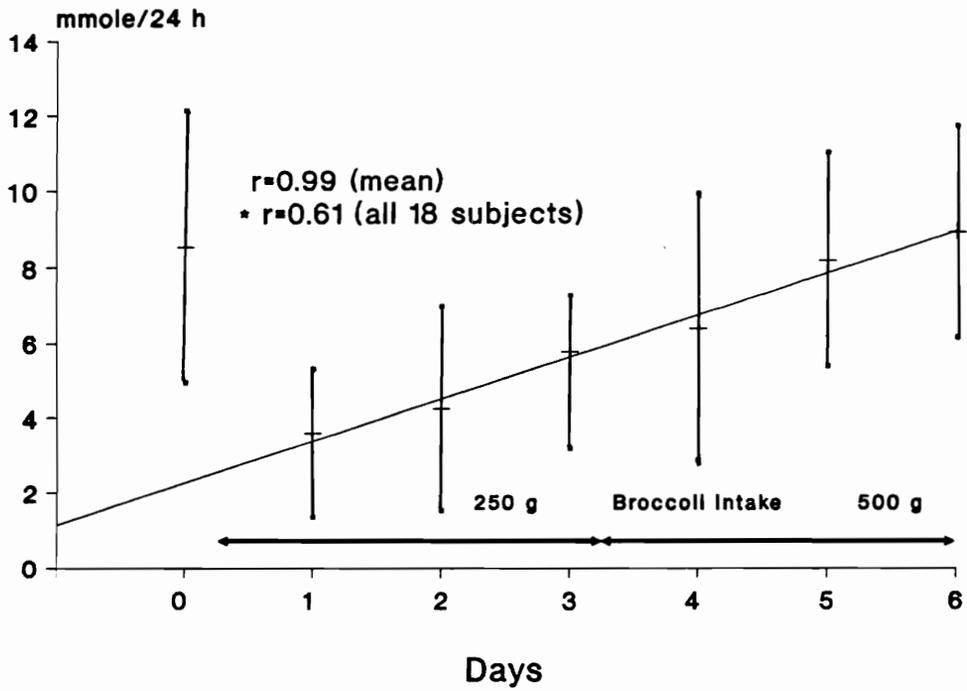
During the broccoli diet treatment, a continuous increase in conjugate excretion was noticed over the six day period. A strong linearity was observed over time ( $P < 0.0001$ ) with a correlation coefficient of 0.61 for all the 18 subjects (Figure 19). The correlation coefficient for the mean values was 0.99. The mean excretion values of the two broccoli levels were different ( $P < 0.0001$ ). However, no differences ( $P > 0.05$ ) in the slope nor the intercept were observed between the two levels of broccoli (Figure 20).

Glucosinolates present in broccoli are readily hydrolyzed by heat as well as the enzyme, myrosinase (thioglucoside glucohydrolase), present in the vegetable (129). The resulting decomposed products consist not only of  $\beta$ -D-glucose and an organic aglycone moiety such as isothiocyanate, thiocyanate, and indole, but also inorganic sulfate. Therefore, in order to determine if the sulfur content in the broccoli had any affect on sulfoconjugate excretion, free sulfate excretion was observed in relation to conjugate excretion profile. Figure 21 is a profile of a free (inorganic), total and conjugate sulfate excretion. The difference between total and free sulfate would be



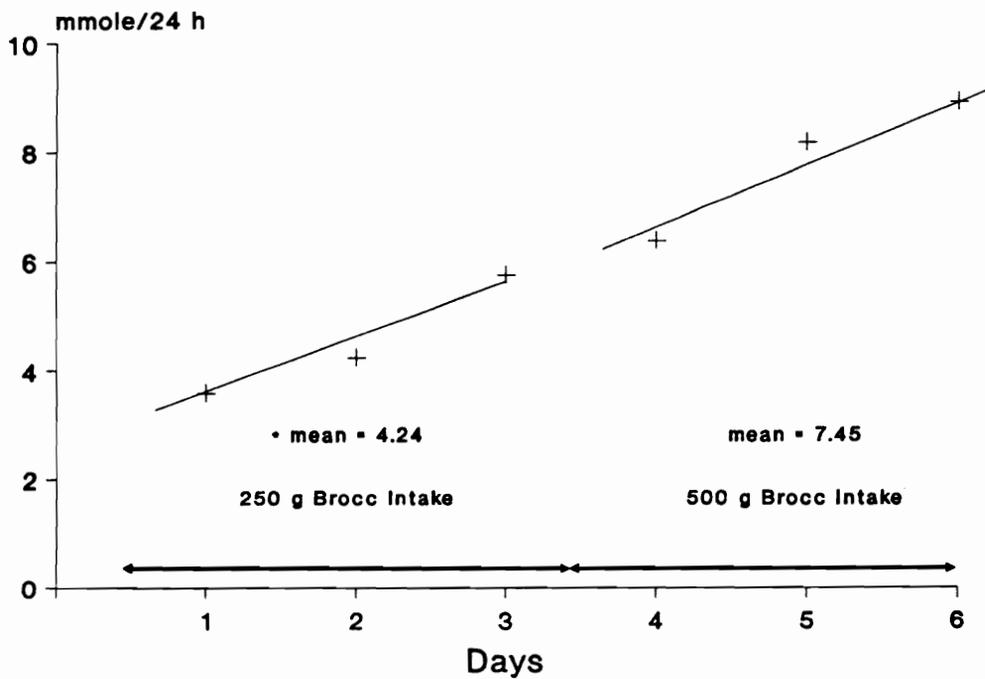
ND = normal diet      ———— interquartile 25-75%  
 LD = liquid diet      \*      at least 1.5X out of quartile  
 BD = broccoli diet      □      at least 2.5X out of quartile

Figure 18. The median urinary sulfoconjugate excretion of adult males. (n=18)



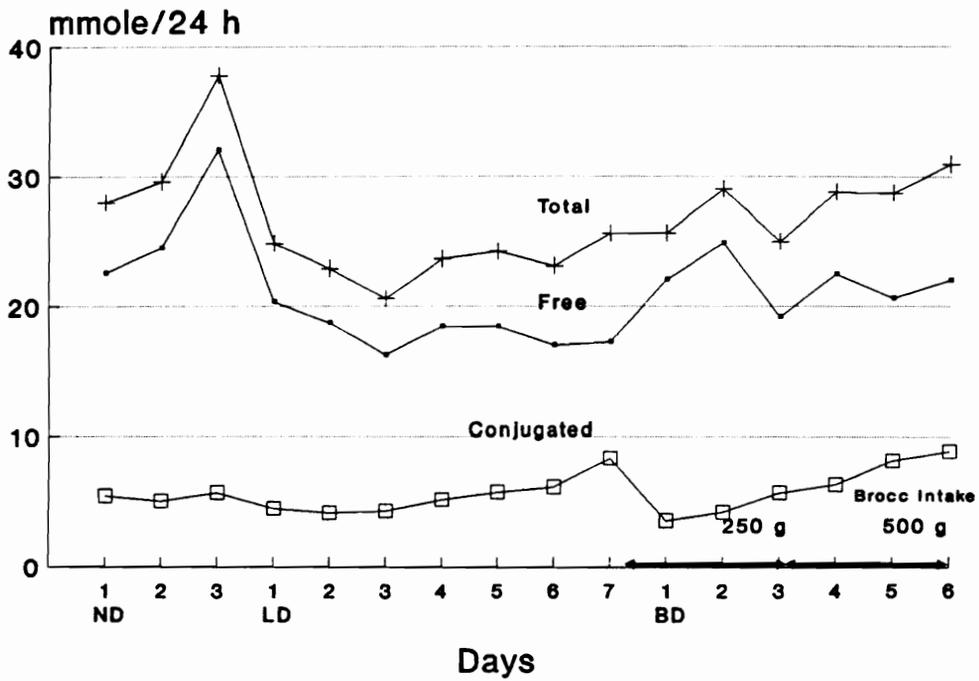
\* A linear increase of excretion level was observed ( $P < 0.0001$ ).

Figure 19. The effect of dietary broccoli on mean( $\pm$ SD) urinary sulfoconjugates in adult males.



\* Mean excretion values of the two levels were different ( $P < 0.0001$ ).  
 Intercepts and slopes of the two levels were not different ( $P > 0.05$ ).

Figure 20. The effects of different levels of dietary broccoli on mean urinary sulfoconjugate of adult males. (n=18)



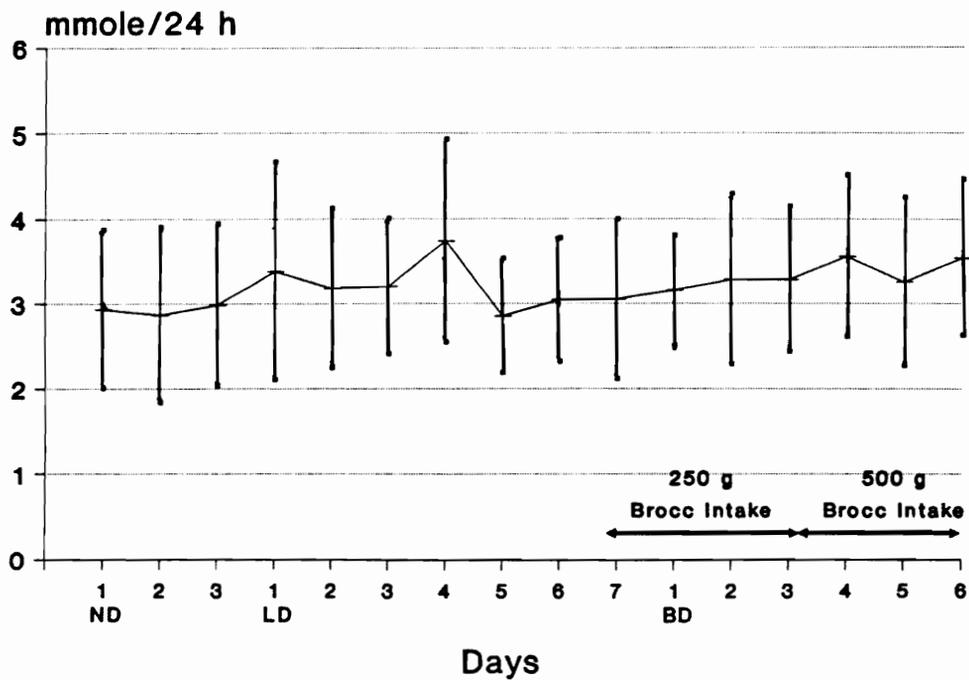
ND = normal diet  
 LD = liquid diet  
 BD = broccoli diet

Figure 21. The mean urinary sulfate excretion of adult males (n=18).

bound sulfate and is represented as conjugated sulfate. On the average, the liquid diet lowered the free sulfate excretion level by 32% compared to that of the normal diet. The 250 g broccoli diet increased free sulfate excretion by 28% and 44% at BD1 and BD2, respectively, compared to that of LD7. However, no apparent increase was observed thereafter, even when broccoli intake was doubled from 250 to 500 g. The free sulfate excretion appeared to be stabilized with continuous broccoli intake. Sulfoconjugate, on the other hand, increased steadily during the dietary broccoli period.

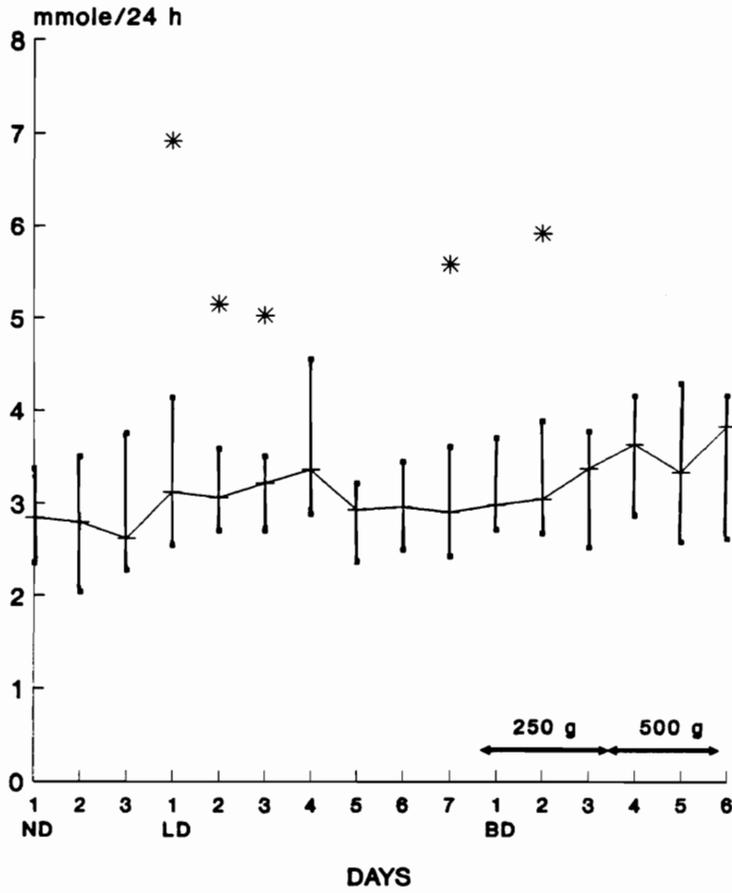
### **C. Glucuronides**

Figure 22 is a profile in the mean glucuronic acid conjugate excretion over time. The profile of the median values is shown in Figure 23, and was very similar to that of the mean. Among the four urinary conjugates, glucuronides were least affected by dietary treatment. The excretion level at LD7 (3.05 mmole/24 h) was practically the same as the average excretion of ND (2.97 mmole/24 h). With dietary broccoli, a slight increase was observed, resulting in a 19% increase at BD6 compared to the average excretion level of ND. No significant ( $P > 0.05$ ) linear trend was observed within the broccoli diet treatment.



ND = normal diet  
 LD = liquid diet  
 BD = broccoli diet

Figure 22. The mean( $\pm$ SD) urinary glucuronide excretion of adult males. (n=18)

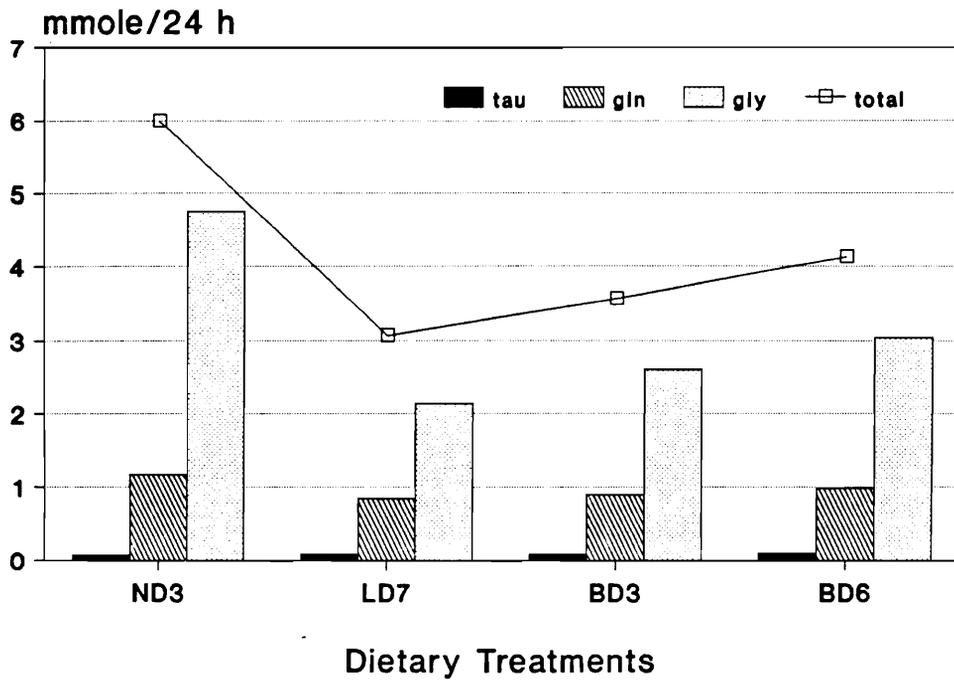


ND = normal diet      ———— interquartile 25-75%  
 LD = liquid diet      \*      at least 1.5X out of quartile  
 BD = broccoli diet

Figure 23. The median urinary glucuronide excretion of adult males (n=18).

#### **D. Amino Acid Conjugates**

Figure 24 represents the changes in mean amino acid conjugate excretion over time. The relative contributions of glycine, glutamine and taurine are also shown. Unfortunately, a 16 day profile was not obtained for the amino acid conjugates. For practical reasons, only excretion levels at the end of each diet treatment, i.e., ND3, LD7, BD3(250g), and BD6(500g), were determined. Furthermore, 18 subjects were randomly divided into 3 groups of 6, then, the samples within each group were pooled for the amino acid analysis. At LD7, total amino acid conjugate excretion was lowered by 49% from that of ND3. With the broccoli diet, an apparent increase of 16% was noticed at BD3 and an increase of 35% at BD6. However, neither increase was statistically significant when compared to LD7 ( $P > 0.05$ ). The amino acid conjugate level at BD3 (3.56 mmole/24 h) and BD6 (4.12 mmole/24 h) continued to be lower than that of ND3. Among the amino acid conjugates, glycine showed the highest excretion levels, followed by glutamine. Also, the glycine conjugates were more responsive to the diet changes than were the glutamine conjugates. For example, glycine decreased 54% by LD7, whereas glutamine decreased only 29%. With the broccoli diet, a 43% increase was observed for glycine at BD6, but only a 17% increase was noticed for glutamine. Taurine excretion levels were negligible for all dietary treatments.



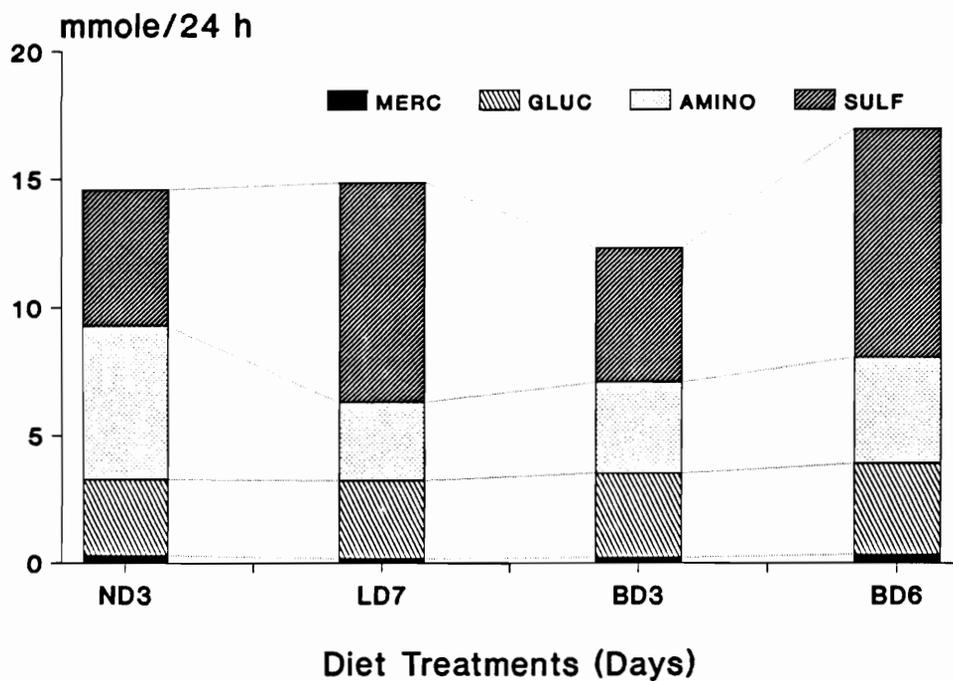
ND3 = 3rd day normal diet  
 LD7 = 7th day liquid diet  
 BD3 = 3rd day broccoli diet 250 g  
 BD6 = 6th day broccoli diet 500 g

Figure 24. The mean urinary amino acid conjugate excretion of adult males (n=3; 18 subjects were divided into 3 groups of 6. The samples within each group were pooled for the analysis.)

## **E. Total conjugate excretion**

The sum of four conjugates and their percent distributions at the end of each diet treatment are shown on Figure 25. A broccoli intake of 500 g (BD6) had the highest total excretion level, whereas a broccoli intake of 250 g (BD3) had the lowest. It appears that with an intake of 250 g, the total conjugate excretion level decreased. However, this was due to a substantial decrease in sulfoconjugate alone, which was the most excreted urinary conjugate. If sulfate is not included, LD7 has the lowest total conjugate excretion, and a rise is observed with 250 g broccoli. In terms of percent distribution, amino acids were the major conjugates excreted at ND3 (41%), but sulfate predominated at LD7, BD3, and BD6.

The inter- and intrasubject variations of conjugates at the end of each diet treatment are shown in Table 9. For amino acid conjugates, variations were not determined since the conjugate values were determined using pooled samples. Overall, intersubject variation was higher than intrasubject variation. The intersubject variation observed during the normal diet was lowered by the 7th day of liquid diet. Intrasubject variation decreased by the 10th and 13th days of liquid diet, i.e., BD3 and BD6, respectively. It appears that consuming the liquid diet lowered the intersubject variation of urinary conjugates. The intrasubject variation also decreased, but only with prolonged use of the liquid diet treatment. Furthermore, the addition of broccoli diet apparently did not increase intrasubject variation.



ND3 = 3rd day normal diet  
 LD7 = 7th day liquid diet  
 BD3 = 3rd day broccoli diet 250 g  
 BD6 = 6th day broccoli diet 500 g

Figure 25. Distribution of urinary conjugates in adult males (n=18).

Table 9. Inter- and intrasubject variation in urinary excretion of conjugates (n=18)

Conjugate	Variation	Dietary Treatment			
		Normal Day 3	Liquid Day 7	Broccoli	
				Day 3 250 g	Day 6 500 g
Sulfate	inter	68 *	42	54	40
	intra	44	43	45	27
Mercapturate	inter	49	40	30	24
	intra	26	31	19	15
Glucuronide	inter	33	29	26	28
	intra	17	20	11	12

\* Coefficient of Variation (%)

Table 10 summarizes the mean excretion levels of each conjugate at the end of each diet treatment. Overall, sulfate conjugates showed the highest excretion level followed by the amino acid conjugates and glucuronides. Mercapturate excretion levels were the lowest. Since it was estimated that at the 7th day of liquid diet (LD7) a stable or baseline level of conjugate excretion would be established, excretion levels on the broccoli diet were compared to that of LD7. Among the four conjugates observed, mercapturates and sulfoconjugates showed significant changes with dietary broccoli. For mercapturates, 250 g broccoli (B3) resulted in a 35 % increase ( $P < 0.005$ ) in conjugate excretion levels over that of LD7. At the end of the 500 g broccoli diet (B6), a 110% increase ( $P < 0.0001$ ) was observed. For sulfoconjugates, an unexpected change in excretion level was observed at LD7. The LD7 excretion level was 61% higher than that of ND3. However, no statistical significance was noted due to the large standard deviation. BD3 substantially decreased (39%,  $P < 0.0001$ ) the excretion level of sulfoconjugates. However, by the time of BD6, the excretion level was comparable to that of LD7.

Table 10. The Effect of Dietary Treatments on Urinary Conjugate Excretion of Adult Males (n=18)

Diet	Conjugates (mmole/24 h)				
	Sulfoconjugate	Glucuronides	Mercapturates	Amino Acid Conjugates	
Self-Selected Day 3	<sup>a</sup> 5.28±4.08	2.98±0.96	0.29±0.19	5.99±0.45	<sup>d</sup>
Semisynthetic Day 7	8.52±3.59	3.05±0.94	0.16±0.05	3.06±0.18	
+ Brocc 250g Day 3	<sup>b</sup> 5.19±2.03	3.29±0.85	0.22±0.06	3.56±0.42	<sup>c</sup>
+ Brocc 500g Day 6	<sup>e</sup> 8.91±2.78	3.54±0.92	0.34±0.09	4.12±0.48	<sup>b,f</sup>
<sup>a</sup> Mean±SD					<sup>d</sup> Different than LD7 (P<0.0001)
<sup>b</sup> Different than LD7 (P<0.0001)					<sup>e</sup> Different than BD3 (P<0.0001)
<sup>c</sup> Different than LD7 (P<0.0005)					<sup>f</sup> Different than BD3 (P<0.0005)

## DISCUSSION

The present study showed that a diet containing broccoli increased the urinary excretion of mercapturates. A significant intercept change between different levels of dietary broccoli suggests that average increases of 1.3 and 2.1 fold of urinary mercapturates from 0, 250, to 500 g broccoli reflect a dose response and not a time effect of the 250 g broccoli diet.

Several studies have shown that cruciferous vegetables, either whole or isolated glucosinolate extracts, induced several enzymes and increased co-substrates levels in animal tissues (14,16,109,110,112,115). Examples include glutathione-S-transferase, dehydrogenases and GSH levels, all of which are directly or indirectly involved in glutathione conjugation (Refer to Literature Review, Section B for a more detailed review). Therefore, it would seem that an increased glutathione conjugation, due to enzyme induction and an increased GSH level may have contributed to the increased incidence of glutathione conjugation. However, it is also equally likely that other nonnutrient compounds in the broccoli may have increased the excretion of mercapturates. In other words, a rise in urinary mercapturates may be due to metabolism of the absorbed compounds from the broccoli as well as an increase in the rates of metabolic conjugation. A combination of these two factors, thus, may have resulted in the increased level of urinary mercapturates.

Dietary broccoli also increased the urinary excretion of sulfoconjugates. The average increase of 1.8 fold of sulfoconjugate from 250 to 500 g broccoli, however, is not necessarily an effect of dose since no statistical difference in the slope nor the intercept was observed between the two levels of broccoli. It is possible that a cumulative effect of feeding only 250 g broccoli over the six day period may have resulted in a continuous rise.

To date no incidence of sulfotransferase induction has been identified (130). Thus, the continuous rise in sulfoconjugates over the 6 days of dietary broccoli observed in the present study does not appear to be a result of enzyme induction. The sulfate group for conjugation comes mostly from the diet as inorganic sulfate or indirectly as organic sulfate. There is evidence to show that when sulfate intake is not limiting sulfoconjugation increases (42,44). Broccoli contains glucosinolates, a family of compounds the hydrolysis of which results in inorganic sulfate (129). Therefore, it is possible that there was an increasing supply of cosubstrate sulfate during the course of the 6 days of broccoli treatment. According to Hele (131), when inorganic sulfate intake is increased, a great increase in urinary output of sulfate occurs in dogs. However, upon addition of xenobiotics such as phenol, or indole, the amount of free inorganic sulfate in urine decreased, and was replaced by a large increase in conjugated sulfates. A similar result was obtained in our study. The intake of dietary broccoli increased free urinary sulfate excretion initially. As intake of broccoli continued, the free urinary sulfate decreased. Furthermore, the decrease

was offset by an increase in conjugated sulfates. It appears that broccoli was not only a source of sulfate, but also a source of xenobiotics which were subject to sulfation. For example, indole, one of the substrates used in the Hele study (131), is a substance known to be present in broccoli. The unexpected decrease in the excretion of sulfate conjugates from LD7 to BD1 remains unexplained.

A few studies have indicated a possible induction of glucuronidation by cruciferous vegetables. For example, human acetaminophen metabolism was stimulated by the intake of cruciferae (17), which was partially due to enhanced glucuronidation. Also, when an isolated indole compound was examined for its effect on aflatoxin metabolism, a significant increase in biliary aflatoxicol glucuronide was observed (132). The implication is that the conjugating enzyme, UDP-glucuronosyltransferase was being induced. However, the results from the present study did not show any significant changes in glucuronate conjugate excretion with dietary broccoli.

Many functional groups capable of glucuronidation can be sulfated as well. Thus, glucuronide and sulfate pathways are regarded as competing pathways. It is believed that factors such as xenobiotic dose levels and kinetic parameters of the enzymes determine the balance between sulfation and glucuronidation. In general, sulfotransferases have a higher affinity (low  $K_m$ ) for their substrates than do UDP-glucuronyltransferases, while glucuronidation has a higher capacity (high  $V_{max}$ ) for substrates. This implies that at high levels of xenobiotics, glucuronidation predominates, whereas at low levels the relative importance of sulfation increases. Usually,

humans, such as the subjects in the present study, are exposed to low doses of xenobiotics. Thus, sulfation would be expected to be quantitatively important for metabolism. Furthermore, there is evidence to show that when sulfate sources are not limited, the ratio of glucuronide:sulfate decreases (42,44). Assuming that the liquid diet contained nutritionally adequate amounts of sulfur/sulfate, and also that broccoli is an additional source of sulfate (from glucosinolate), it appears that sulfation predominated and glucuronidation remained fairly constant.

The present study indicates that a broccoli diet has no effect on urinary amino acid conjugates in man. However, it appears that the normal diet contained a compound, or compounds, which caused an increased amino acid conjugate excretion. To date, there are no studies which suggest an induction of amino acid conjugation by cruciferous vegetables. However, there have been several attempts to induce amino acid conjugation in humans using salicylate. For instance, when 26 volunteers were pretreated with aspirin for 3 days, salicyluric acid formation increased (133). Also, when rheumatic patients were treated with aspirin, an increased rate of salicyluric acid synthesis was observed primarily in the first two weeks of treatment (134). It has been postulated that autoinduction may help explain why some patients fail to maintain adequate serum salicylate concentrations during therapy (134). Among the three amino acids observed in our study, glycine and glutamine were the major urinary conjugated amino acids. Furthermore, glycine was relatively more responsive to dietary change compared to glutamine. Conjugated amino acids excret-

ed by normal adults consist primarily of hippuric acid (benzoylglycine) and phenylacetylglutamine (74,75). Hippuric acid accounts for 65-75% of the glycine conjugate (76), whereas phenylacetyl glutamine accounts for 50% of the glutamine (73). Benzoic acid, a compound from which a hippuric acid is formed, is ingested or produced in vivo. Benzoic acid and its derivatives also occur naturally in plants and are thus readily available in the diet. The origin of phenylacetic acid is less clear. It has been postulated that it is an endogenous end-product of phenylalanine metabolism (135).

Dietary broccoli given with the liquid diet increased the urinary excretion of mercapturates and sulfoconjugates, but did not significantly change the excretion of glucuronides and amino acid conjugates. The excretion of mercapturates was shown to be in a dose-dependent manner with the three levels of the dietary broccoli (0, 250, 500 g). The excretion of sulfoconjugates may not be dose-dependent, and instead may have been due to the effect of time. Overall, it appears that the effect of dietary broccoli was more apparent as a consequence of the implementation of the liquid diet, which reduced variability.

The subjects total caloric intake and macronutrient distribution remained constant throughout the dietary regimen. Therefore, it is unlikely that weight loss or change in the body composition have contributed to the urinary conjugate excretion. The consumption of dietary fiber, however, increased 1 to 2 fold during the broccoli diet. High intake of dietary fiber may have increased its binding to various

compounds such as conjugates excreted in the bile. Thus, it is possible that increase in dietary fiber may have influenced the biliary conjugate excretion. However, whether it may have affected the urinary conjugate excretion is speculative and is not clear with the present study.

Several compounds in broccoli have been identified to alter xenobiotic metabolism, perhaps the addition of these compounds in isolated form to the control diet would be an interesting future study. Of further interest would be an examination of the levels of urinary conjugates after broccoli treatment has been withdrawn.

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**APPENDIX A**  
Survey Questionnaire

**TOXICOLOGICAL PROFILE ASSESSMENT STUDY  
SELECTION CRITERIA SURVEY**

NAME

---

AGE

---

HOME PHONE

---

BUSINESS PHONE

---

LOCAL ADDRESS

---

SOCIAL SECURITY NUMBER

---

CODE NUMBER

---

**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. NoSpecify: \_\_\_\_\_  
\_\_\_\_\_
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 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

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.)  
 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 your 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 if you have any additional questions:

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

**APPENDIX C**

**Daily Food Intake Record**



## **APPENDIX D**

### **Body Weight Changes of the Subjects**

Subject	ND1	ND2	ND3	LD7	BD1	BD2	BD3	BD4	BD5	BD6	Mean ± SD
01	74.9	74.2	74.5	75.1	74.5	74.6	74.6	75.4	74.9	74.5	74.7 ± 0.4
02	83.1	82.6	83.0	82.2	82.0	82.7	82.6	82.9	82.5	81.5	82.5 ± 0.5
05	98.0	98.2	98.3	96.0	95.8	95.7	95.8	95.4	95.1	94.5	96.3 ± 1.4
06	69.5	69.7	69.5	68.5	68.5	69.5	69.3	68.7	69.0	68.2	69.0 ± 0.5
07	79.1	79.5	79.5	78.2	77.1	77.1	76.8	78.1	77.3	77.3	78.0 ± 1.0
10	94.5	94.3	94.4	93.2	93.4	92.7	93.0	93.6	92.1	92.1	93.3 ± 0.9
11	76.0	76.6	75.3	76.1	76.2	76.2	75.3	76.2	75.5	75.5	75.9 ± 0.5
12	76.1	77.2	76.0	76.2	75.8	75.2	75.6	76.7	75.7	75.7	76.0 ± 0.6
13	65.9	67.0	65.7	65.8	65.9	65.6	66.3	66.3	66.4	66.0	66.1 ± 0.4
14	96.1	96.1	95.7	94.4	94.2	93.9	94.1	94.9	94.6	94.7	94.9 ± 0.8
16	58.9	58.9	59.1	58.0	58.5	58.7	58.5	59.0	58.9	58.9	58.7 ± 0.3
17	68.5	68.7	68.4	67.6	67.6	67.6	67.9	67.5	66.4	66.4	67.7 ± 0.8
19	86.6	86.5	86.4	86.1	86.1	85.8	85.5	85.7	84.7	84.8	85.8 ± 0.7
20	66.3	66.0	65.8	65.4	65.4	65.5	65.0	65.3	65.3	65.3	65.5 ± 0.4
24	77.2	76.6	76.8	76.3	77.6	76.4	77.9	78.1	77.5	77.5	77.2 ± 0.6
27	74.4	73.4	73.4	75.1	73.5	74.9	73.6	74.0	73.7	73.3	73.9 ± 0.7
28	69.2	69.2	69.3	69.6	69.8	69.1	69.9	69.9	69.9	69.1	69.5 ± 0.4
29	72.2	73.1	73.2	71.0	71.0	70.8	71.2	71.6	71.7	71.7	71.8 ± 0.8
Mean	77.0	77.1	76.9	76.4	76.3	76.2	76.3	76.6	76.2	75.9	76.5 ± 0.4

Body Weight (KG) changes of 18 subjects over experimental period.

## **APPENDIX E**

### **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 F**

### **Determination of Urinary Mercapturates**

## DETERMINATION OF MERCAPTURIC ACIDS

### SAMPLE PREPARATION

1. Pipet 5 ml of urine sample for centrifugation (5 min at 1,300 X G; DAMON/IEC, HN-SII).
2. Aliquot  
1 ml of urine (sample)  
1 ml of deionized water (di-H<sub>2</sub>O) (blank)

At one go, run 9 samples in triplicates and 1 blank

Screw cap polypropylene test tubes are required. (95X16.8 mm, 13 ml capacity Cat No. 55.518; Walter Sarstedt, Inc., Princeton, NJ)

### DETERMINATION OF FREE SULFHYDRYL GROUPS (PRE-HYDROLYSIS)

1. Start heating water bath to a temperature of 60° C. While waiting for the water bath to reach the desired temperature, make a fresh solution of 5% NaBH<sub>4</sub>. (2.5 g NaBH<sub>4</sub> in 50 ml di H<sub>2</sub>O)

Prepare NaBH<sub>4</sub> with ice cold (refrigerated) di-H<sub>2</sub>O to minimize bubbling. Also keep NaBH<sub>4</sub> solution in the ice bucket.

2. Add 1 ml of the freshly prepared 5% NaBH<sub>4</sub> solution to aliquoted samples and blank. Mix and heat in a water bath (60° C) for 15 min. The purpose of this step is to reduce any -S-S- groups which may be in the urine.

Keep the test tubes capped properly to minimize oxidation of the free -SH groups.

4. After 15 min, remove test tubes and cool in an ice bath for 10 min.
5. Excess NaBH<sub>4</sub> must be destroyed as follows. (NaBH<sub>4</sub> interferes Ellman's

reagent).

Decomposition must be done carefully and slowly since poisonous gas  $BH_3$  is evolved and the solution tends to foam.

- i) add 1 ml acetone to test tubes - shake and wait for 5 min.
- ii) add 1 ml 2.5 M HCl - shake and wait for 10 min: make sure that there is no effervescence, which signifies that incomplete destruction has taken place.
- iii) add 0.2 ml, 1% (w/v) metaphosphoric acid, mix and wait 2 min.

When bubbling stops, invert test tubes to decompose any traces of  $NaBH_4$  on the side of tubes.

## COLOR REACTION

1. For urine sample:  
2.00 ml 0.25 M (pH 7.52) phosphate buffer  
0.30 ml Ellman's reagent (DTNB)  
0.75 ml sample from test tubes
2. For urine color blank:  
2.00 ml 0.25 M phosphate buffer  
0.30 ml di H<sub>2</sub>O instead of Ellman's reagent  
0.75 ml sample from test tubes
3. For Ellman's Reagent color blank:  
2.00 ml 0.25 M phosphate buffer  
0.30 ml Ellman's reagent (DTNB)  
0.75 ml deionized water

Cover the cuvettes and invert

Wait 10 - 20 min before reading ABS.

Read the Abs at 412 nm.

Timing needs to be the same for all samples, because the characteristic yellow color continues to increase in intensity with time.

#### DETERMINATION OF -SH GROUPS AFTER HYDROLYSIS

1. Aliquot fresh 1 ml of each sample, standard, blank as prehydrolysis. Add 1 ml of 5 M NaOH to these samples. The NaOH cleaves the -S- bonds of the mercapturic acid as well as -S-S- to liberate free -SH groups.
2. Bubble nitrogen through this solution for 15 sec and cap very tightly to prevent oxygen from 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 5 min. Amount of HCl is critical. An excess of HCl will interfere with the color development and cause variabilities between replicates.

#### COLOR REACTION

Same as pre-hydrolysis. See color reaction on previous page.

#### STANDARD CURVE

Standard solution (N-Acetyl Cysteine) spiked with a urine sample was used for pre- and posthydrolysis standard curve development.

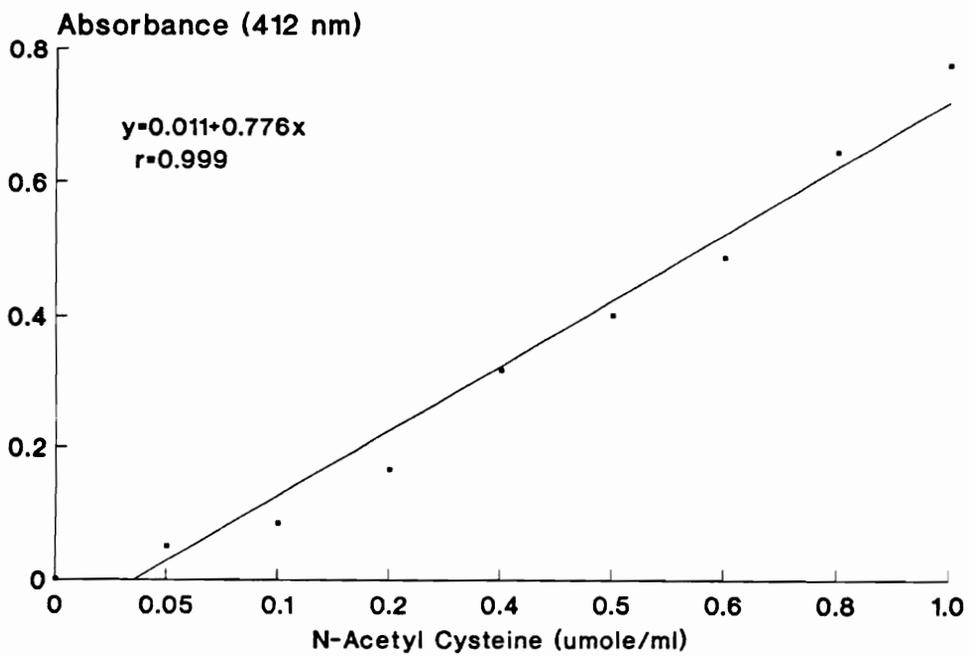
Standard concentration:

0.05, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0 umole/ml

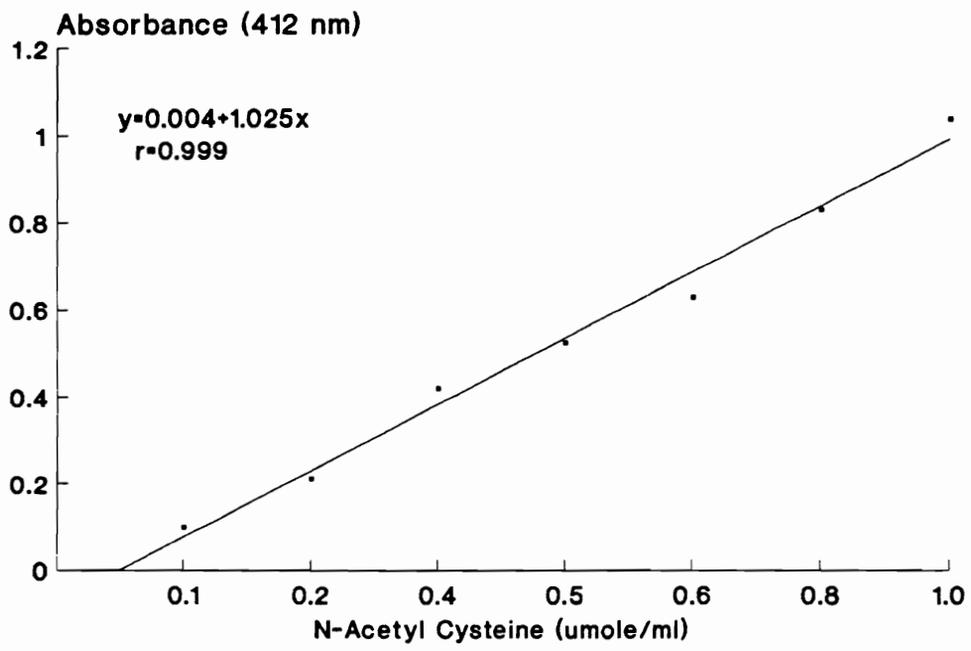
Aliquot

0.5 ml N-Acetylcystein (NAC), 0.5 ml urine (spiked standard)  
1 ml Phenol Mercapturic Acid (to check if hydrolysis occurred)

Phenol Mercapturic Acid (PMA) was used to varify the hydrolysis step.



Standard Curve for free -SH



Standard Curve for bound -S-X

## PREPARATION OF REAGENTS

0.25 M Phosphate buffer solution pH=7.52

$\text{Na}_2\text{HPO}_4$  (MW=141.96) sodium phosphate dibasic  
dissolve 35.49 g of above into 1 L di water

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (MW=137.99) sodium phosphate monobasic  
dissolve 17.2485 g of above into 500 L di water

Add monobasic to dibasic until solution reaches pH=7.52

DTNB (5.5'-dithiobis(2-nitrobenzoic aci)) reagent (0.4mg/ml) =Ellman's reagent

To make total vol of 500 ml DTNB

0.2 g DTNB

5.6 g Sodium Citrate

Make up to 500 ml with di water

Store at 4°C. This can be used up to 13 weeks.

### META-PHOSPHORIC ACID Solution

Dissolve 1 g meta-phosphate into 100 ml di water

### Phenol Mercapturic Acid Stock Solution:

50 mM (50  $\mu\text{mol}/\text{ml}$  = 0.05 M)

Dissolve 2.390 g PMA in 100 ml di water

### N-Acetyl Cystein Stock Solution:

100mM (100 $\mu\text{mole}/\text{ml}$  = 0.1 M)

Dissolve 1.64 g in 100 ml di water

## **APPENDIX G**

### **Determination of Urinary Sulfoconjugates**

## DETERMINATION OF SULFOCONJUGATES

Sulfate to be measured is in the form of Barium Sulfate ( $\text{BaSO}_4$ ). The actual component being measured by the atomic absorption spectrophotometer is Barium. Sulfate is only indirectly measured through Barium, thus the molecular weight difference between Ba and  $\text{SO}_4$  has to be taken into account in the calculation.

### A. Inorganic sulfate analysis:

1. Pipette duplicates of 1 ml urine sample into polypropylene conical centrifuge tubes (15 ml capacity, Falcon Brand, Cat No. C3917-16). For standard curve, prepare 1 ml of three different concentrations of standard, i.e., 0.5, 1.0, 2.0 ppm ( $\text{Na}_2\text{SO}_4$ ) to run along with each urine sample run. Standard does not require duplicates.
2. Add to these, 3 ml deionized water, 5 ml barium chloride (1.78% (w/v) aqueous  $\text{BaCl}_2$ ), 0.18 ml (5-7 drops) conc.HCl. Solution becomes cloudy. Wait 10 min.  
White ppt will form at the bottom of the tube.
3. Centrifuge the samples cold ( $9^\circ\text{C}$ ) at 5,000 x G for 30 min.(DAMON/IEC, DPR-6000 centrifuge)
4. Discard the supernatant and wash the pellet with 5 ml deionized water, twice.

Centrifuge and discard the supernatant with each wash.

Supernatant can be poured out with caution. Do not attempt to drain all the liquid, because it will disturb the precipitate.

After the last wash, remove all the supernatant by pouring out some and using a pasteur pipet to remove the remaining liquid.

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 with 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. Make sure to aspirate the standards ( $\text{Na}_2\text{SO}_4$  see reagent preparation on the following page) before the samples so that the sample concentration can be calculated from the standard curve.

Atomic Absorbtion Spectrophotometer Setting  
(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 ( $\text{N}_2\text{O}$ )	Oxidant Flow (L/min): 5.3
Fuel: Acetylene ( $\text{C}_2\text{H}_2$ )	Fuel Flow (L/min): 6.5
Energy: 70	

B. Sulfoconjugate analysis:

1. Take 2 ml urine into screw cap (teflon lining) pyrex culture tube (20X125 mm Corning Glass, Corning, NY., Cat No. 9825-20). Add 1.5 ml fuming nitric acid into the sample tube, and hydrolyze for 24 hr at 110° C oven. Make sure to seal the sample tightly with screw caps. Label the caps as well as the tubes.
3. After heating allow the tubes to cool.
4. Under a fume hood, open the tubes and heat them in a sand bath (Fisher, Hi-Temp Bath M160) at 300-350° C until the nitric acid evaporates.

This is the step where most of the sulfate loss might occur. Thus, make sure the temperature is not too high (390° C) because destruction of sulfate might occur. At the same time the correct temperature is

needed to evaporate nitric acid in the shortest amount of time. For sand bath, dial # 225-250 was used.

5. After the end of the evaporation step, a white/yellow dried residue remains at the bottom of the tubes. Reconstitute the residue by adding 2 ml deionized water.

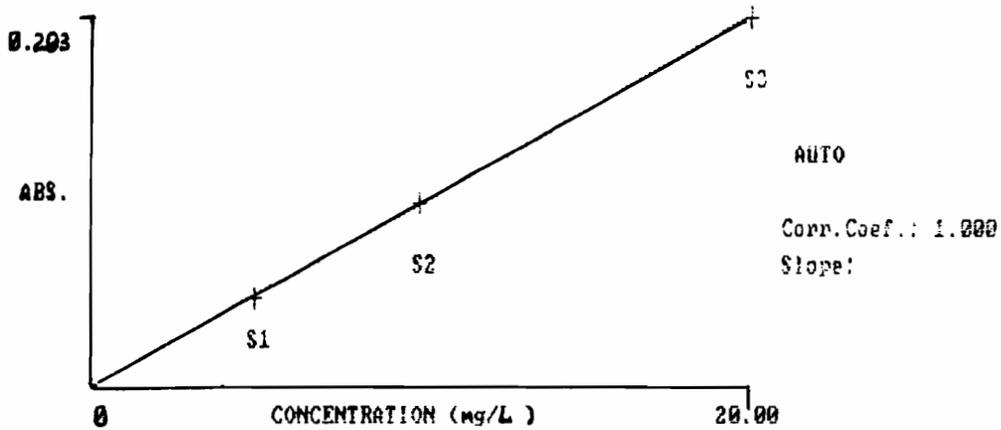
Put in the water bath (60-70° C) for 12-15 hr to dissolve ppt.

Vortex. Pipette 1 ml of the reconstituted sample into the polypropylene centrifuge tubes (Falcon C3917-16).

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

Dehydroisoandrosterone 3-sulfate (a steroid sulfate) DIAS is used to determine the recovery of hydrolysis.

DIAS should be run along with each batch of sample analysis.



Standard curve for sulfoconjugate

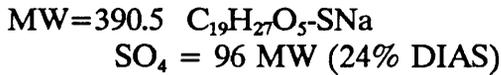
## REAGENT PREPARATION FOR SULFATE ANALYSIS

### 1. Ammonium EDTA:

10 g EDTA, free acid form, dissolved in 100 ml concentrated ammonium hydroxide containing 19.07 g potassium chloride and brought up to the final volume 1000 ml with deionized water

### 2. 1.78% (w/v) BaCl

### 3. Dehydroisoandrosterone 3-Sulfate (DIAS)



1 g total amount as bought from Sigma was dissolved in 100 ml di-water. This gives 1 g/100 ml = 10,000 ug/ml = 10,000 ppm DIAS, and 2,400 ppm SO<sub>4</sub>. (1 ppm = 1 mg/L)

This solution will be further diluted to 250 X by the time it is read in the AA, the expected concentration of SO<sub>4</sub> in the DIAS is;

$$2,400 \text{ ppm} / 250 = 9.6 \text{ ppm}$$

### 5. Na<sub>2</sub>SO<sub>4</sub>

20 ppm of Sulfate

20 ppm = 2 mg/100 ml  
multiply by dilution factor of 250 gives 500 mg/100 ml.

1 mole of sulfate react with 1 mole of barium.  
Barium MW = 137.34  
Therefore, 500 mg/100 ml Barium = 3.64 mmoles /100 ml

3.64 mmoles/100 ml sulfate = 349.498 mg /100 ml  
(Sulfate MW = 96)

Sulfate is 67.56% of Na<sub>2</sub>SO<sub>4</sub> (MW=142.04 g)

Therefore 517.3 mg/100 ml of Na<sub>2</sub>SO<sub>4</sub> is needed to give 349.498 mg/100 ml Sulfate.

Concentration of 5, 10, 20 ppm were prepared from the above stock solution.

## **APPENDIX H**

### **Determination of Urinary Glucuronides**

## DETERMINATION OF GLUCURONIDES

### Urine Preparation

1. Mix well 5 ml aliquot of urine.
2. Boil for 20 min in a 100° C water bath. Cool.
3. If precipitate appears, transfer to centrifuge tubes (polystyrene graduated conical 15 ml capacity, Fisher Brand, Cat No. 05-527-45) and centrifuge for 5 min at 1,300 X G (DAMON/IEC, HN-SII).
4. Supernatant will be used.  
For blank, deionized water will be used

### Glucose Oxidation

This step removes glucose interference by converting free glucose to gluconic acid which is insensitive to NR reagent:

1. Into a 25 ml volumetric flask, add 2.0 ml of the prepared urine sample or a blank. To this add 1.9 ml of 0.1 M acetate buffer (pH 5.6) and 0.1 ml glucose oxidase Type V (G-6891, approx 1,000 units per ml, Sigma Co).

To dilute the concentration of urine which absorbance is  $>10$ , add 1.0 or 0.5 ml of urine as necessary and make up the volume to 2 ml with deionized water.

Gluconic acid is in fact naphthoresorcinol positive at high conc, This possible interference can be eliminated by preparing a blank of glucose.

A blank, using 1 mg/ml glucose solution and 1 ml of deionized water should be prepared in the same way as the urine samples.

2. Incubate the volumetric flasks in a water-bath at 37° C for 45 min. Continuously aerate the mixture by blowing air into the flask. Use a

pasteur pipet attached to a manifold connected to the air compressor. The air needs to be filtered through glass fiber.

3. After incubation most of the liquid evaporates. Bring the volume up to 25 ml with deionized water. Mix well.

#### Decomposition of Free Glucuronic Acid

This step converts free glucuronic acid to a naphthoresorcinol insensitive products

1. Remove three, 1.5 ml aliquots of the diluted urine and blank and transfer to glass test tubes containing 0.5 ml of 2N NaOH and 1.0 ml distilled, deionized water.
2. Boil this mixture for 20 min. Allow to cool to room temperature in an ice bath.

#### MNR Procedure: Color Development

1. After cooling, add 2.0 ml MNR reagent and 2.0 ml concentrated HCl.
2. Vortex, boil for 30 min, and then cool on ice water bath briefly until tubes cool down (3-5 min). The reaction product is pink-bluish in color and is stable for about 1 hr.
3. Vortex, add 3.0 ml ethyl acetate to the test tubes. To extract the reaction product shake vigorously (by hand) for 10 sec. Consistent vigorous shaking is very important for maximum absorbance.
4. Pipette the acetate layer into a quartz cuvette and read the absorbance at 564 nm against a blank. Reproducibility among triplicates should be  $\leq 5\%$ .
5. Determine the concentration of conjugated glucuronic acid from the standard curve.

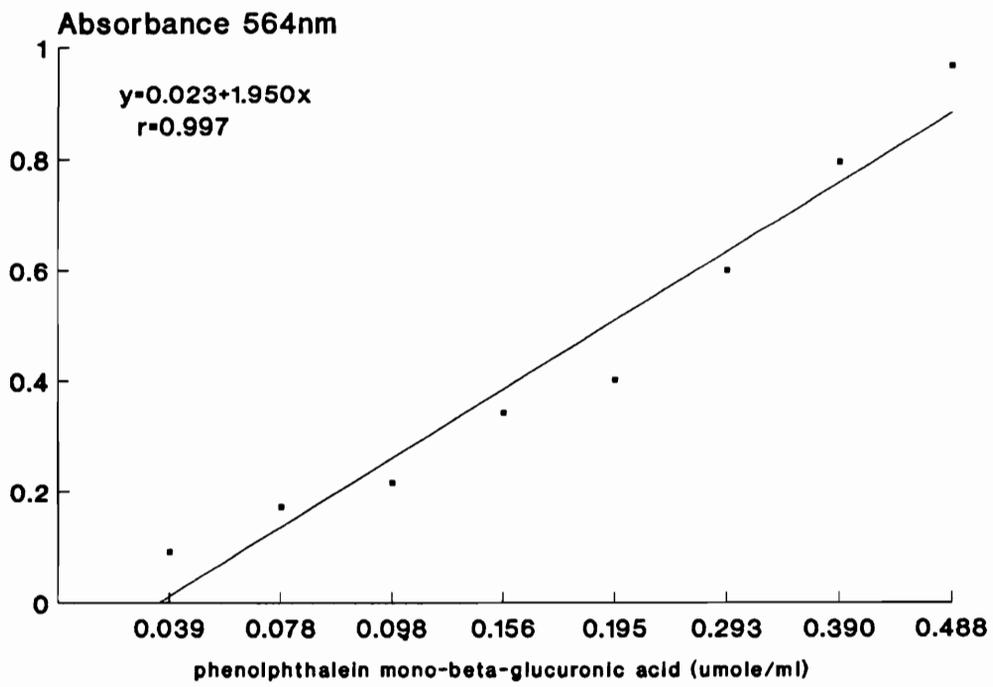
### Standard Curve Preparation

1. Perform the glucose oxidation step as above with a urine sample. Prepare enough urine sample since each standard concentration needs to be spiked.

Phenolphthalein mono- $\beta$ -glucuronic acid (P-GIDE) is the reference compound used for the development of standard curve.

Concentrations of P-GIDE used are:  
0, 20, 40, 50, 80, 100, 150, 200, 250  $\mu\text{g/ml}$ .

2. Add 1 ml of different concentrations of P-GIDE to test tubes containing a urine sample (run in duplicates). For 0  $\mu\text{g}$  P-GIDE, add 1 ml deionized water instead of P-GIDE.
3. Add 0.5 ml of 2 N NaOH to the test tubes. Now proceed from the free glucuronic acid decomposition step.



**Standard curve for glucuronides**

## Reagent Preparation

### Modified naphthoresorcinol reagent (MNR):

1. In a 500 ml volumetric flask, dissolve 2.0 gm of naphthoresorcinol (NR) using 250-300 ml deionized, distilled water.
2. Add drop-wise 0.5 N NaOH (about 5 ml is required) to adjust the pH to 8-8.5. 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 a pH 2-2.5 is obtained. Solution should turn light yellow. Acidification helps to stabilize the reagent.
4. Add sodium bisulfite (1.0 gm  $\text{Na}_2\text{S}_2\text{O}_5$ ) and adjust volume to 500 ml with deionized water. Bisulfite also stabilizes the reagent.
5. Filter the solution 2-3X and refrigerate at 0-5° C until ready to use. Protect from light by wrapping volumetric flask with aluminum foil.

The reagent will remain stable for 3-7 days.

### 0.1 M Acetate Buffer pH 5.6

A: 0.2 M acetic acid, glacial = 1.155 ml bring up to 100 ml

B: 0.2 M sodium acetate ( $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ ) = 13.60 gm bring up to 500 ml

Mix A = 48 ml, B = 452 ml and bring up to 1000 ml.

Adjust to pH 5.6 by adding either A or B accordingly.

### 100 ml of 0.5N NaOH

50 ml of 1N NaOH and make up to 100 ml in volumetric flask.

10%(w/v) meta-phosphoric acid

10 gm phosphoric acid in 100 ml H<sub>2</sub>O.

## **APPENDIX I**

### **Determination of Urinary Amino Acid Conjugates**

## DETERMINATION OF AMINO ACID CONJUGATES

### Urine Preparation

1. Dilute the urine sample 1:1 with internal standard solution (Norleucine): 200 ul urine, 200 ul Norleucine.
2. Filter the sample through Millipore filtration device. (Ultrafree MC-10,000 NMWL, low binding cellular filter) Molecular weight cut off is 10,000.
3. Spin at 1,500 g for 15 min.
4. Collect the filtrate and place it in labeled micro-vials. Freeze (-20°) until derivatization.

### Free Amino Acid Analysis

1. Aliquot 25 ul of the samples (duplicate) into labelled sample tubes (6X50 mm).

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.

2. Add 10 ul of Redry solution to each sample tube, vortex, place in a reaction vial and dry to 50 mTorr. Let sit at 50 mTorr for 15 min.
3. Repeat the above step.
4. Add 20 ul of derivitization reagent to each tube. Vortex the tubes, place them in the reaction vials and place the reaction vials in the vacuum station WITHOUT 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°).

5. Using a Hamilton syringe, add 100 ul of diluent to each sample tube and vortex. Place the sample tubes in the small microcentrifuge (Speed-Vac) and spin for 30 sec on low (1,200 rpms).
6. Remove the supernatant for HPLC injection.

Solvent system: Eluent 1 and Eluent 2

Detector used wavelength 254 nm

#### Hydrolysis (For amino acid conjugate analysis)

1. Prepare the urine samples as described on the previous page.
2. Aliquot 25 ul of sample, using a Hamilton syringe, into sample tubes. Dry to 50 mTorr.
3. Add 100 ul of 6N HCl with 1% phenol to the bottom of the reaction vial.

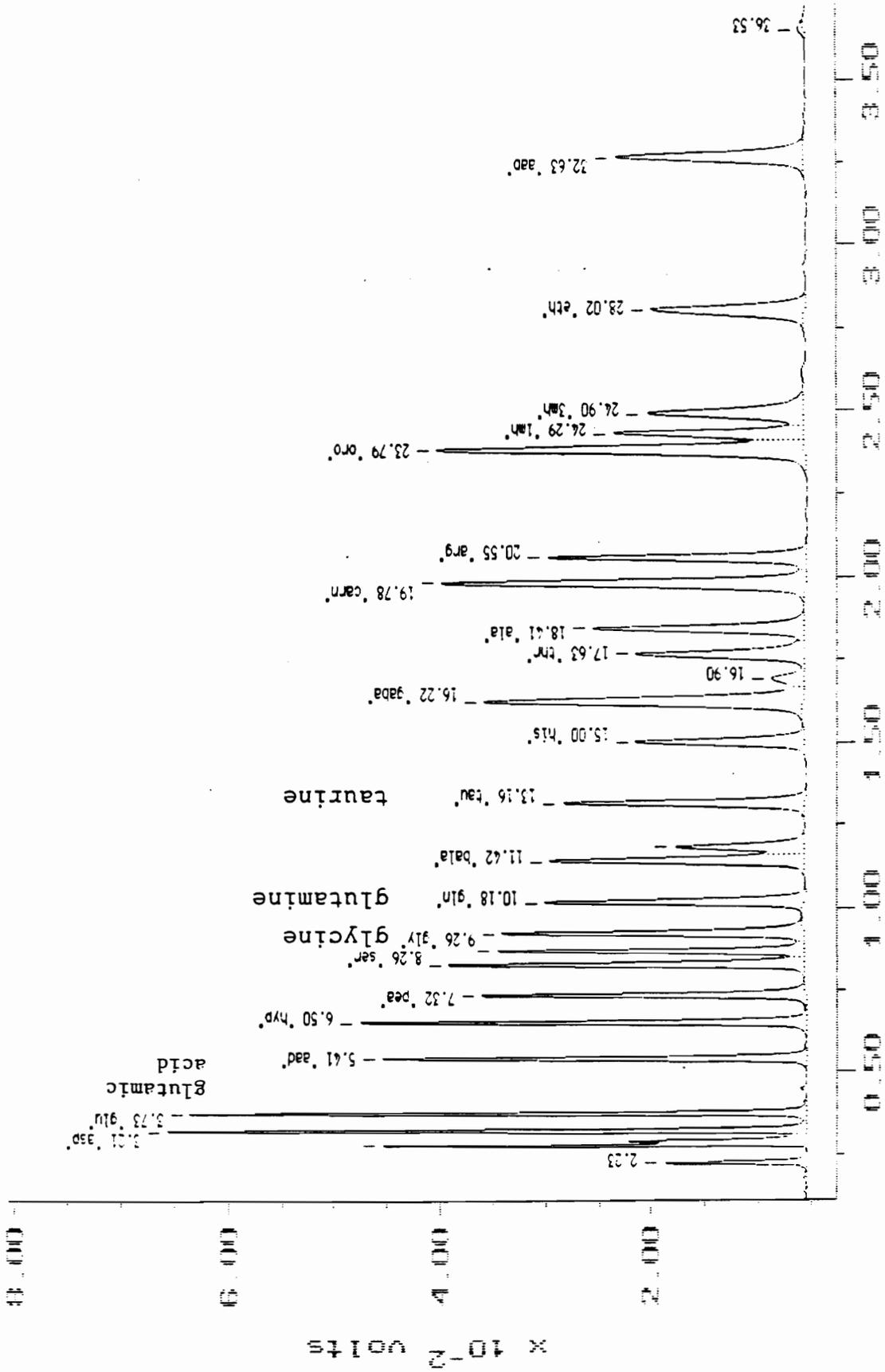
Be careful not to get any HCl inside the sample tubes.

Purge the reaction vial with nitrogen and cap.

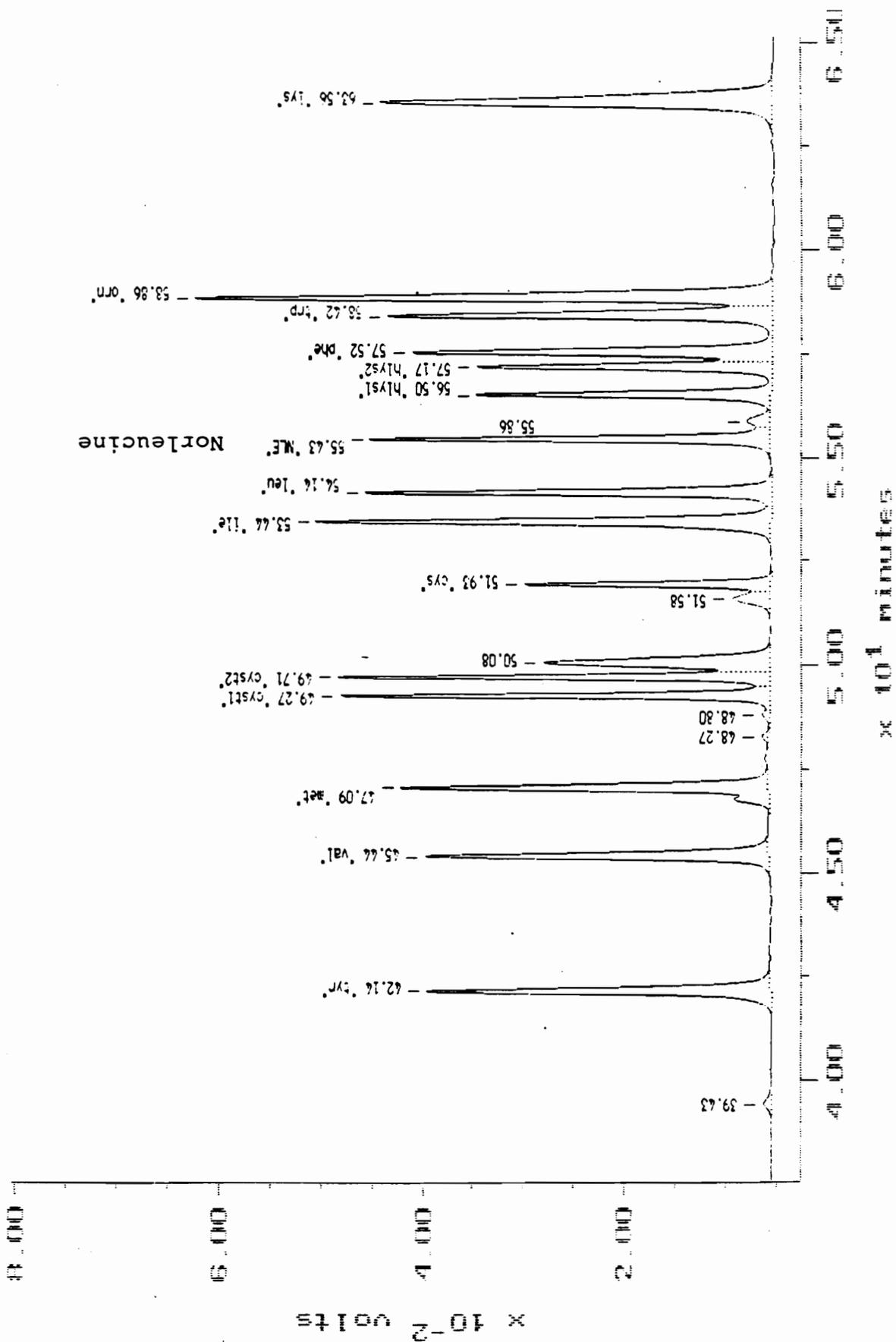
4. Place the vial in the vacuum station and pull a slight vacuum (1-2 Torr) for about 20 sec or until you see the HCl start to boil. Close the valve and remove it from the vacuum station.
5. Purge the vial with nitrogen using the small hole on the top and the valve (do not UNSCREW the cap). Quickly close the valve.
6. Repeat steps 3 and 4 twice more.
7. Place in the vacuum station and pull to a vacuum of 1-2 Torr.

8. Place the reaction vials in an oven for 24 h at .
9. After 24 h, remove the vials from the oven being careful not to get HCl condensation in the samples (Cool them on a slant). Wipe off the tubes and put them in a clean, regular reaction vial.
10. Place in the vacuum station and dry to 50 mTorr.

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



STANDARD CHROMATOGRAMS: Physiological standard containing internal standard Norleucine (CONT)



STANDARD CHROMATOGRAMS: Physiological standard containing internal standard Norleucine

## Reagent Preparation

### A. Internal Standard Solution

0.4 mM Norleucine in 0.1 N HCl  
(Norleucine MW = 131.2,  
weigh out 0.0052 gm and bring up to 100 ml with 0.1N HCl)

### B. Diluent

Can be purchase premade from Waters (# 88119)  
Weigh out 710 mg of disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) and add 1 L of deionized water pH to 7.4 with 10% phosphoric acid ( $\text{H}_3\text{PO}_4$ ). Filter. Add acetonitrile to make a 5% by volume concentration. Store at 4°C.

### C. Redry

A 2:2:1 ratio of methanol (HPLC grade):1M sodium acetate: TEA (triethylamine).

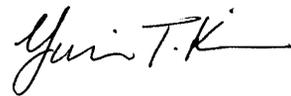
0.8 ml methanol  
0.8 ml 1M sodium acetate  
0.4 ml TEA

### D. A 7:1:1:1 ratio of methanol (HPLC) : TEA : $\text{DiH}_2\text{O}$ : PITC(pheylisothiocyanate)

490 ul methanol  
70 ul TEA  
70 ul  $\text{DiH}_2\text{O}$   
70 ul PITC

## VITA

Yuni was born on March 3, 1963, in Seoul, Korea. She came to the U.S. with her family on April, 1982. In 1986 she received her B.S. degree in Nutrition Research from University of Maryland, College Park Campus. In the Fall of 1986, she enrolled in the Department of Human Nutrition and Foods at Virginia Polytechnic Institute and State University. She earned her M.S. in Human Nutrition and Foods in 1988, and was subsequently awarded her Ph.D. in Human Nutrition and Foods in 1992. Yuni will begin her Postdoctoral Fellowship at the Medical Biology Institute, La Jolla, CA in November, 1992.

A handwritten signature in black ink that reads "Yuni T. Kim". The signature is written in a cursive style with a horizontal line extending to the right from the end of the name.

Yuni T. Kim