THE URINARY EXCRETION OF AMINO ACID CONJUGATES IN FREE LIVING ADULT MALES

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(ABSTRACT)

The objective of this research was to quantitatively assess the urinary excretion of glutamine and glycine conjugates in a free living population of young male adults, in order to establish a profile of detoxication via amino acid conjugation. Also, the effect of certain factors (vegetable, fruit, meat, and charbroiled food intake; tobacco, alcohol, caffeine, and marijuana use; exposure to chemicals and familial cancer incidence) on the urinary excretion of the amino acid conjugates were investigated. Three consecutive 24 hour urines were collected from 40 subjects who complied with a specific collection protocol. The urine samples were analyzed using a HPLC amino acid analyzer. The mean conjugated glutamine excreted was 1.30 mmole/24 hr or 8.74×10^{-2} mmole/mmole creatinine/24 hr. The mean value for urinary conjugated glycine was 3.91 mmole/24 hr or 26.38×10^{-2} mmole/mmole creatinine/24 hr. For glutamine conjugate excretion, vegetable, fruit, alcohol, chemical exposure and marijuana use showed marginally significant differences among their subgroups. For glycine conjugate excretion, meat, caffeine, chemical exposure, cancer and marijuana use showed mar-

ginally significant differences among their subgroups. An analysis of variance revealed a large degree of between-subject(inter) and within-subject(intra) variability. The coefficients of variation for glutamine and glycine for inter-variability were 51.1 and 53.4%, respectively, whereas the coefficients of variation for intravariability were 37.3 and 31.4%, respectively. Probably, the large variability masked any effects of diet, environment or genetics on the observed urinary conjugated amino acid excretion.

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CHAPTER I: INTRODUCTION

Humans are continually exposed to an imposing number and variety of chemical compounds through a combination of environmental, occupational, dietary , social, and medicinal sources. Some of these chemical compounds are indispensible to life processes, while others are foreign to the normal energy-yielding and nutrient-utilizing metabolism of the human (1). Such non-nutritive foreign compounds are referred to as xenobiotics (1-3).

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

site (3,5).

Although generalizations are tenuous at best, the extremely diverse and complex metabolism of xenobiotics is conveniently classified as a bi-phasic process (1-8). Phase I metabolism involves oxidative, reductive, and hydrolytic reactions in which one or more functional polar groups are exposed or introduced on the parent compound (2-5). Such polar groups, including; hydroxyl, amino, carboxyl, and epoxides, provide a reactive site at which phase II metabolism can occur (5-8). Hence phase I metabolism not only tends to enhance the water solubility of the compound, but also generates a metabolite which is suitable as a substrate for phase II metabolism (7,8). A complete discussion of phase I metabolism may be found in Hodgson and Dauterman (4), and in Alvares (5).

During phase II metabolism, the parent xenobiotic and/or its phase I metabolite(s) undergo synthetic reactions which involve covalent conjugation to an endogenous molecule or grouping, referred to as the conjugating agent (6-8). There are numerous classes of conjugation reactions recognized; however, the eight major ones are glucuronidation (glucose conjugation), sulfation, methylation, acetylation, cyanide detoxication, glutathione conjugation, and amino acid conjugation (6,7). In general, the result of such conjugation reactions is the production of rela tively non-toxic, water soluble acids which are readily available for elimination from the body at a considerbly more rpid rate

than either the parent xenobiotic or its phase I metabolite(s) (6-8). Indeed, the majority of excretion products from xenobiotic metabolism are found in conjugated forms (1). A review of the phase II conjugation reactions is presented by Dauterman (8).

The classification of xenobiotic metabolism as a bi-phasic process erroneously implies a general sequentiality and mutual exclusiveness between the two phases. In fact, as cited by Dutton (9), "there is no direct linkage of phase I and phase II reactions in the body genetically or topologically. The processes develop, and are inducible, independently, and can occur in different areas of the cell." An individual xenobiotic and/or its metabolite(s) undergo multiple phase I and phase II reactions both consecutively and concurrently (1,7). In some cases, phase I alone generates the excreted metabolite(s), illustrating that conjugation is not inevitable (1,6), while on the other hand, direct conjugation to a parent xenobiotic frequently occurs (10). Therefore, as previously stated, generalizations are tenuous at best.

Further clarification becomes necessary when considering the overall significance of xenobiotic metabolism. Since the net result appears to be reduced toxicity and enhanced excretion, the phase I and phase II reactions have been popularily referred to as detoxication mechanisms and the enzymes which catalyze the events, as detoxication enzymes (1-4). However, both phase I and

phase II reactions are capable of metabolic activation, in which case, a biologically inactive compound is converted to one with considerable toxicological activity (1-10). Indeed, there are many noted cases in which the intermediary or final effect of xenobiotic metabolism is intoxication rather than detoxication (5,7). Therefore, more recently, the term biotransformation has been adopted to collectively describe all of the phase I and phase II reactions involved in xenobiotic metabolism, without reference as to the consequences of activation and/or inactivation (2). Nevertheless, for the majority of xenobiotics, the ultimate effect of biotransformation is inactivation and enhanced excretion (10).

Xenobiotics and/or their metabolites are excreted from the body predominantly in the urine, bile, and feces, but also via expired air, perspiration, vomitus, hair, and mammary secretions (2). These pathways of excretion and the various mechanisms employed are by obligation, identical with those involved in the elimination of endogenous waste products (11,12). Hence, it should not be surprising that renal and hepatic pathways of elimination predominate.

Urinary and biliary pathways of excretion are known to be complementary; however, the mechanisms which dictated the principal routes remain unclear (11-14).

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

an organism to a xenobiotic is most critically dependent on the highly complex realm of the phase I and phase II biotransformation. Unfortunately, most toxicological investigations have vigorously focused attention on the phase I biotransfromation, leaving the conjugation reactions relatively neglected (6). From a pharmacological viewpoint, specifically product development, therapeutic effectiveness, and safety, such an emphasis is understandable since that metabolic activation occurs primarily during the phase I biotransformations (5). However, the consequence is a lack of knowledge concerning the multiple variability factors potentially influencing the conjugation reactions as a whole (10).

The significance of conjugation as a detoxication mechanism becomes obvious only when the reaction is impaired due to saturation or to a metabolic defect. In such case the protective effect is voided and the toxicity is manifested in some manner (6,7). Salicylate intoxication is one of the examples. The major route of salicylate metabolism is conjugation with the amino acid glycine to form salicylurate. At a usual low therapeutic dose, salicylate is converted to salicylurate and rapidly eliminated with a half-life of 3-4 hours for adults. However, at a high therapeutic dose or overdosage, salicylurate formation is completely saturated and the half-life is prolonged to about 30 hours. Therefore, not only recovery from an overdosage is very slow, but there is a serious danger of accumulation and toxicity.

Such intoxication is a common medical emergency which carries a significant mortality, especially in young children (15).

From an evolutionary viewpoint, when one compares detoxication capacity and efficiency between lower organisms and more advanced ones, it is the conjugation reactions where improvement is mpost often found (1).

Of the eight major conjugation reactions, amino acid conjugations were the first to be isolated. In the review of detoxication mechanisms and their historical aspects, Williams (16) reported that hippuric acid, which is a glycine conjugate (or benzoyl glycine), was found by Rouelle in 1784 in cow's urine. Williams further noted that later, in 1942, Keller showed that hippuric acid could be formed from ingested benzoic acid.

Although the amino acid conjugates were the first of the so-called detoxication mechanisms to have been described, they are the least well investigated and understood. This lack of attention is probably due to the diversity of amino acids in nature and also the large variations of the amino acids utilized, the animal species involved, and the structures of carboxylic acid being detoxified.

The amino acid conjugation reaction, in general, involves a carboxylic acid compound forming a peptide bond with an amino acid. The amino acids most commonly encountered in conjugation reactions are aliphatic and dietarily non-essential such as glycine, glutamine, ornithine and taurine. Among these four

amino acids, glycine is the most wide spread and versatile in conjugation reactions. It is encountered in all mammals and is utilized for the conjugation of carboxylic acids of diverse structure. By contrast, the other three amino acids conjugates are restricted in terms of either or both their species occurrence or the type of acid that can be conjugated. Taurine conjugation is restricted to arylacetic and cholic acid derivatives, but is relatively widespread in its species occurrence, which includes man. 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. Glutamine conjugation is even more restricted in its occurrence. It is largely confined to the conjugation of arylacetic acids and only by anthropoid species including human (17). Given this overview, glycine, glutamine and taurine appears to be the major amino acids being utilized in man for their conjugation reactions.

As part of an overall research project which has as its principle objective the determination of a profile of the detoxification pathways in the human, this study was designed to specifids conjugates are restricted in terms of either or both their species occurrence or the type of acid that can be conjugated.

CHAPTER II: LITERATURE REVIEW

Almost all the organic nitrogen necessary for the human body is derived from dietary protein. Consequently, amino acids which are basic components of protein would serve an enormous variety of physiological and metabolic functions. This review of literature will primarily be concerned with the functions of amino acids as conjugation agents in the detoxication of xenobiotics.

A. Amino Acid Composition of Urine

There have been some studies regarding amino acid composition of urine. The complete picture of daily excretion of free amino acids in normal adults irrespective of dietary habits can be found in several publications (18,25,26,65).

Figure 1 shows the relative amounts of 23 excreted urine amino acids in a group of normal healthy adults drawn from a paper by Soupart (26). Of these 23 amino acids, 22 are free amino acids and the twenty third is the amino acid derivative taurine. As shown in the figure, among the 23 amino acids, 9 add up to approximately 85-90% of total free amino acid excretion, whereas the other 14 amount to only about 10-15%. Among the 9 amino acids, glycine ranks first (27%) in abundance followed by taurine, histidine, methylhistidine and glutamine (8%). In Table 1, the quantitative data of the 9 amino acids are listed in

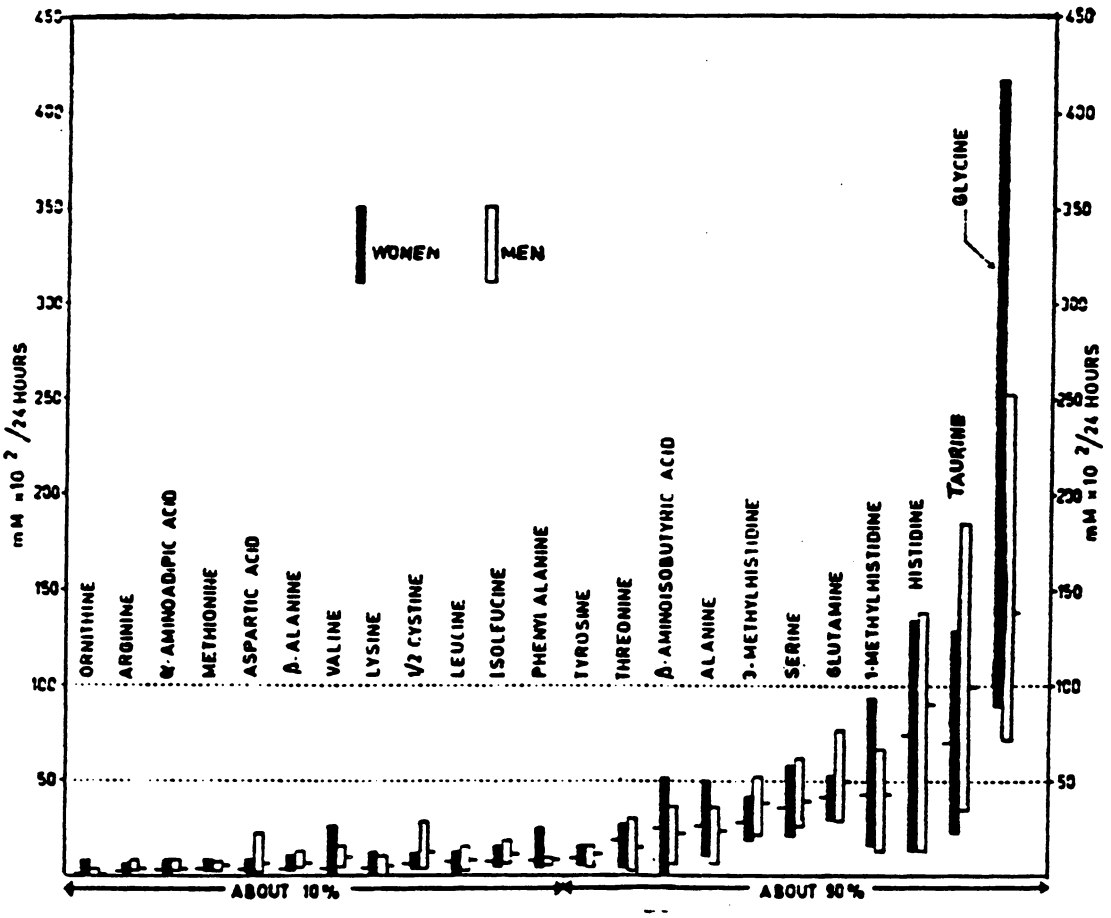


Figure 1. Free urinary amino acids of healthy adults (26)

Table 1. Daily urinary output of 23 free amino acids in 15 normal adult men and women (26)

A. 9 amino acids (85-90% of total)	micromoles / 24 hour	
	Range	Average
Glycine	710-4160	1687
Taurine	220-1850	812
Histidine	130-1370	790
1-Methylhistidine	130- 930	433
Glutamine *		at least 350
Serine	310-620	374
3-Methylhistidine	180-520	323
Alanine	60-500	257
beta-Aminoisobutyric acid	0-500	252
Total		at least 5278
B. 14 amino acids (10-15% of total) Excreted in small amounts (0-300 micromoles) in the average 65 micromoles		

* Minimal values; glutamic acid added to glutamine on a molar basis since freshy voided urine does not contain glutamic acid.

decreasing order of average excretion. In agreement with Stein's chromatographic analysis(18), Soupart's (26) results did not show any glutamic acid value. According to Stein (18), no appreciable amounts of glutamic acid were found in freshly voided normal urine; however, it had been noticed that the glutamic acid content of normal urine increased on standing even at 4 C. The detection of glutamic acid with stored urine was predicted to occur in part from glutamine hydrolysis. Therefore, the glutamine value shown in Table 1 is actually the sum of glutamine and glutamic acid. However, this glutamine value is only a minimal estimation since part of the free glutamic acid may escape determination by partial cyclization into pyrrolidone carboxylic acid which does not react with ninhydrin (27).

The amino acid elution curve obtained by using the Moore and Stein method (28) also elucidated that there are about 40 to 50 different ninhydrin positive substances in normal human urine. Ninhydrin positive substances include free amino acids, substituted free amino acids, or amino acid derivatives in which the amino group stays free, enabling them to react with ninhydrin (29).

B. Acid Hydrolysis of Urinary Conjugated Amino Acids

Many ninhydrin positive substances are acid-stable which

means the peak is unaltered in position and undiminished in area when an acid hydrolysate of urine is chromatographed (10). Upon acid hydrolysis of the urine, however, there was a marked in crease in some amino acids (18), especially glycine and glutamic acid. A marked increase would indicate that a large amount of these amino acids are normally excreted in a conjugated form. On the other hand, Stein (18) reported in his study that there was little or no increase observed for taurine. It thus appears that the major amino acids utilized for conjugation reactions in man are glycine and glutamine.

Conjugated amino acids excreted by normal adults consist primarily of hippuric acid and phenylacetylglutamine (26). This is in agreement with Soupart's (29) statement that, "the bulk excretion of conjugated amino acid is chiefly composed of substances such as hippuric acid or phenylacetylglutamine and even some peptides.". The presence of peptide in urine seems very minute, however, since considerable volumes of urine had to be processed to recognize the presence of peptides (30). Therefore, the contribution of amino acid from peptides after hydrolysis seems insignificant.

Hippuric acid represents a detoxication product of benzoic acid whereas phenylacetyl glutamine represents a detoxication product of phenylacetic acid. According to Stein et al.(69), a normal adult male excretes 1.0 -2.5 gm of hippuric acid per day which accounts for 65-75 % of the observed conjugated glycine in

a 24 hr urine (This value is higher than 0.75 -1.00 gm (18) which Stein previously reported). Phenylacetyl glutamine, which is another predominant conjugate in urine has been found to be excreted under normal conditions by the adult male to the extent of 250 -500 mg per day (69). This accounts for about 50 % of the conjugated glutamic acid in urine. In man, phenylacetic acid is conjugated almost exclusively with glutamine and accounts for 90 % of a 24 hr urine excretion (72).

C. Hepatic and Extrahepatic Distribution of Amino Acid Conjugation

Aromatic and arylalkyl carboxylic acids commonly undergo metabolic conjugation prior to excretion using either a carbohydrate or an amino acid as the conjugating agent. Among mammalian species the carbohydrate is usually glucuronic acid, but in the case of the amino acids, the particular pattern of amino acids used depends upon the individual species (19). As mentioned earlier, glycine, glutamine, and taurine have so far been shown to participate in conjugation reactions in man, and furthermore only glycine and glutamine conjugates were excreted in the significant amount in the urine.

The amino acid conjugates, mainly, appear to be associated with the organs of elimination, such as kidney and liver. The relative importance of the two sites varies, however, with

species and the structure of the acid undergoing conjugation (17). Von Lehman and his coworkers (20) have investigated in man the renal contribution to the total glycine conjugation of aromatic acids. Their research showed that in man some 68% of the glycine conjugation of salicylate is carried out by the kidney with 32% by the liver. The possible significance of extrahepatic sites (kidney) of glycine conjugation was also investigated by Caldwell et al. (21) using human cadaver samples for determining tissue hippuric acid levels. The results showed high hippuric acid formation in both liver and renal tissue, particularly renal cortex. These studies support the view that the kidney can be an important site of glycine conjugation, particularly if one takes into account the high blood flow and therefore high xenobiotic delivery rate to this organ, as well as its intrinsic conjugation activity.

Little is known about the tissue distribution of glutamine and taurine conjugates. Moldave and Meister (22) have detected glutamine conjugating activity in both human liver and kidney, using phenylacetic acid as substrate, but no other tissue was investigated.

D. Reaction Mechanism

Amino acid conjugation is an endergonic peptide bond synthesis reaction which requires an energy-rich intermediate.

In general, there are three types of energy-rich compounds utilized directly in peptide bond formation. These are acyl adenylates, which are acid anhydride derivatives of AMP (adenylic acid), acyl derivatives of coenzyme A (CoASH), and acyl phosphate (31). For the amino-acyl-adenylate and acyl phosphate compounds, the energy rich bond is a carboxyl group - acid anhydride bond, whereas for the acyl CoASH compound, it is a thioester bond. In all cases, however, activation consists of forming an energy-rich bond, and the source of energy to form these high energy bonds is derived from breaking a pyrophosphate bond of ATP.

The type of energy rich compound used in peptide bond formation varies with the kind of peptide synthesis. In the formation of an amino acid conjugation, the peptide bond is formed by the reaction of an acyl CoASH compound with an amino group. The energy in the thioester bond of CoASH, which is available for peptide bond formation, originates from ATP. Then the acyl-S-CoA, an activated intermediate, reacts with an amine to form a peptide bond. Initially, researchers surmised that the amino acid conjugation reaction involved a high-energy intermediate which probably involved an aromatic acid (17). However, Chantrenne (23) later disproved this hypothesis by demonstrating that benzoyl phosphate, an aromatic acid, was not the high energy compound involved, but instead it was the coenzyme A (CoASH) derivative. In 1953, Schachter and Taggart (24) demonstrated synthesis, and suggested the following reaction mechanism for

glycine conjugation reaction.

$$R\ COOH + ATP \longrightarrow R\ CO\ AMP + PPi$$

$$R\ CO\ AMP + CoA\ SH \longrightarrow R\ CO\ SCoA + AMP$$

$$R\ CO\ SCoA + H\ N\ CH\ \ COOH \longrightarrow R\ CONH_2CH_2\ COOH + CoA\ SH$$

R COOH: carboxylic acid compounds

$H_2N\ CH_2\ COOH$: glycine

The mechanism of glutamine conjugation reaction appears to be analogous to that of glycine involving coenzyme A (CoASH), ATP, and an activated derivative of the carboxylic acid except that glutamine, instead of glycine, is conjugated with the carboxylic acid. Our understanding of the glutamine conjugate mechanism is based largely on the study done by Moldave and Meister (22) who proposed the conjugation reaction of phenylacetic acid to phenylacetyl glutamine.

In general, most metabolic conjugations involve the interaction of the xenobiotics with the conjugating agent in which one of the two substrates is in the form of an activated nucleotide (17). In most cases, the conjugating agent is the one in an activated form; however, in the amino acid conjugation, as shown in

the above reaction, the carboxylic acid substrate itself forms a thioester with coenzyme A to become activated. The thioester then reacts with an amino acid. This is one of the unique characteristics which differentiates amino acid conjugation from the other conjugation reactions in phase II detoxication metabolism.

E. Site of Reaction

Another distinct feature of amino acid conjugation besides the reaction mechanism 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 (17). In their early work, Moldave and Meister 1957 (22) partially purified glutamine N-phenylacetyl transferase from the mitochondrial fractions of human liver. Also, more recently, glycine N-acyl transferase was purified from human mitochondria (32) which further supports the view of mitochondria as the site of amino acid conjugation. The mitochondria are the main site of production of acetyl-CoA fragments derived from carboxylic acids during the course of metabolism of amino acids and fatty acids. Because of potential acylating functions, the excessive amounts of acyl-CoA derivatives must be inactivated. If not disposed of, the acyl-CoA derivatives might conceivably participate in the non-enzymatic

acylation of mitochondrial acceptor molecules such as amino acids, peptides, and protein and thus alter function. Since there is a need for mitochondria to be protected against excessive amounts of reactive acyl-CoA esters, and amino acid conjugation is one way of removing these compounds, it is reasonable to assume that the site of amino acid conjugation lies within the mitochondria.

F. Factors Affecting Amino Acid Conjugation

Xenobiotic metabolism, including amino acid conjugation, is highly influenced by an extremely large number of variability or "host" factors (37-41). These factors can be broadly classified as environmental, physiological and genetic. However, there is a complex and dynamic interrelationship between all variability factors, irregardless of such classification (40,41). This complex and dynamic interrelationship makes it virtually impossible to examine the impact of an isolated variable on the rate and pattern of metabolism, let alone on the overall toxicity of a xenobiotic compound (40,41). Therefore, it is understandable why environmental, physiological and genetic factors influencing the amino acid conjugation mechanism have not been thoroughly investigated.

So far, little is known about factors affecting glutamine conjugation <u>in vivo.</u> Yet, glycine conjugation has been shown to

be influenced by some factors, such as, age, disease and interindividual variations.

Studies of hippuric acid formation by Irjala (42) and Caldwell et al. (21) have shown that glycine conjugation appears to be at a low level in the fetus and neonates. This conjugation, however, develops rapidly and results in progressively increased excretion throughout the first year of life (43). Other studies concerning geriatric subjects, as compared to young adults, revealed that there was an apparent decrease in the glycine conjugation with increasing age. Stern et al. (44) indicated that the activity of glycine conjugation decreased in response to a dose of benzoic acid. Later the reason appeared to be a reduced availability of glycine in old age, since administration of the amino acid restored the ability to convert benzoic acid to hippuric acid.

The extent of glycine conjugation of a carboxylic acid appeared to be influenced by a number of dysfunctions, particularly those involving the liver. At one time, the ability to conjugate an aromatic acid with glycine was used as the basis of a test of liver function (45). Later, the test fell into disuse since the occurrence of an impaired glycine conjugation did not always correlated with the extent or nature of liver damage. In vitro studies confirmed that other dysfunction such as kidney disease could also markedly affect glycine conjugation. Caldwellet al. (21) found that post-mortem kidney samples from a case of

systemic lupus erythematosus failed to convert benzoic acid to hippurate. Kidney is a major site of glycine conjugation of certain substrates. Therefore, it is no surprise that some drugs show a slow rate of metabolism in kidney disease. For example, the antitubercular drug p-aminosalicylic acid (PAS) was shown to have an infinitely long half life in an uremic patient, and this was attributed to the slower rate of glycine conjugation in this condition (46).

There also are marked interindividual variations in the extent of glycine conjugation of salicylic acid and isonicotinic acid in man. Alpen et al. (47) found that in four patients, the extent of conjugation of an oral dose of salicylic acid with glycine varied from 0-50% of the dose. Peters et al. (48) also have observed individual variations in the extent of glycine conjugation of isonicotinic acid both when the acid was given as such and as its precursor isonicotinic acid hydrazide. Nevertheless, neither study determined whether the source of these variations was genetic or environmental.

G. Justification

Humans are continually exposed to an imposing number and variety of xenobiotics through a combination of environmental, occupational, dietary, social and medicinal sources. Although xenobiotic metabolism is highly complex, phase II conjugation

reactions are most often successful in terms of detoxication (1).

Since the development of foreign compound metabolism as a distinct subdiscipline in the 1950's, the phase I reactions, most notably the oxidations, have attracted the most attention. Since phase II reactions involve high energy intermediates, research has been conducted mainly in intermediary metabolism rather than biochemical pharmacology and toxicology (10). Nevertheless, it is the phase II conjugation reactions that are most often successful in terms of detoxication by ending the biological activity of the compound whereas phase I reactions can produce active or reactive metabolites (49,50).

This view of our current knowledge of xenobiotic metabolism suggests the development of a profile of the detoxification capability of the body by measuring the end products excreted in the urine, such as conjugates. Renal excretion demands high water solubility and a high degree of ionization with no requirement for lipophilic/hydrophilic balance. Consequently, small, highly ionized, highly polar conjugates are preferentially excreted in the urine, whereas large conjugates tend to have sufficient lipophilic character to favor hepatic biliary elimination. Unlike large glucuronides and glutathione conjugates that are excreted principally in the bile, amino acid conjugates of xenobiotic acids are mainly excreted in the urine (10). From the observation of the excretion of aromatic compounds in the rat, Hirom et al. (13) concluded that the major route of elimination

for the compounds of less than 350 daltons is urine. Since hippuric acid and phenylacetylglutamine, the most abundant form of conjugated amino acid, have molecular weights of 179 and 193, respectively, their primary excretory route would be urine. Thus it appears that urinary excretion of amino acid conjugates can be used as a biological parameter of detoxication without too much concern relative to fecal excretion.

Considering the previous research (56,66) which has been conducted to examine the urinary excretion of glucuronic acid and sulfate conjugates of xenobiotics as a parameter of xenobiotic exposure, it is evident that the need exists for establishing the pattern of urinary amino acid conjugate excretion on a population basis. In order to propose a biological threshold limit of exposure and to establish a meaningful interpretation of the health significance of such a threshold, it is essential to first determine the range of excretion of urinary amino acid conjugates in a normal population and to take into consideration the overall effect of a number of critical variables assessed on this range. Hence, the purpose of this research will be to quantitatively assess the urinary excretion of glutamine and glycine conjugates in a free-living population of young male adults, in order to establish a profile of detoxication via amino acid conjugations.

The primary objectives of this research will be:

1. to develop and refine the methodology for quantification of

glycine and glutamine conjugates;

2. to determine the range and interindividual variation of glycine and glutamine conjugate excretion on a twenty-four hours basis;

3. to determine the range and intraindividual variation of

glycine and glutamine conjugate excretion over three consecutive days for a subgroup of the sampled population; and

4. to examine the interindividual population profile (objective 2) for correlations between the amount of glycine and glutamine conjugated excreted and the exposure to certain variables which could affect such a profile; for example, dietary patterns, caffeine, tobacco, alcohol, marijuana and also genetic factors such as cancer incidences among family members.

CHAPTER III: Methodology

A. Subject Recruitment

Preexperimental surveys, food frequency questionnaires and urine collections were completed two years ago. Details of these methods can be found in "The Urinary Excretion of Conjugated Glucuronic Acid in Healthy Male Volunteers" by P.S. Murano (56). Following is the summary of the procedure.

Healthy adult males who were not taking medications and not under a physician's care, were recruited for the study. Each subject was required to complete the preexperimental survey (Appendix A) prior to urine collection, in an attempt to quantitate the frequency of exposure to caffeine, alcohol, tobacco, social drugs, medications, and environmental xenobiotics. Also, each subject was required to complete the food frequency questionnaire (Appendix B) to obtain presumptive evidence of dietary adequacy and descriptive information on the dietary patterns of the population.

Three 24-hour urine samples were collected from each subject. A 24-hour period was defined as beginning with the collection of the second voiding on day one, through the first voiding on day two. The total volume and pH of 24-hour urine samples were determined daily on delivery of the urine sample. Then, each specimen was subsampled into several aliquots and immediately frozen (-20°C) for future analysis.

B. Variable assessment

1. **Dietary Factors** Data obtained from the food frequency questionnaire were used to ascertain the frequency of consumption of various foods. Frequencies of vegetable, fruit, and meat consumption were categorized as follows:

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

Moderate Range (M) = up to once/day

High Range (H) = more than once/day

Vegetable, fruit and meat intakes were chosen as a potential variable affecting conjugated amino acid excretion because diets high in these food items have been associated with an increase in drug metabolism, thus enhanced drug clearance in human (57,58,63).

Charbroiled meat intake also was chosen as a potential variable affecting conjugated amino acids and was categorized as follows:

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

High Range (H) = >3 times/month

Charbroiled meat intake was chosen as a potential variable because, components such as benzo(a)pyrene in charbroiled foods have been shown to induce cytochrome P-450, an electron transport system involved in phase I detoxication reactions (60).

2. **Nondietary (Environmental) Factors** Preexperimental

survey data were used to ascertain each subject's exposure to various substances. Frequencies of exposure to tobacco, alcohol, marijuana, caffeine, chemicals (chemicals may be organic solvent such as xylene, benzene, gasoline, carbon tetrachloride, acetone, insecticides, and herbicides) were classified as follows:

Low (L) = smoking less than 1 cigarette/day
alcohol less than 3 times/month
marijuana less than once/week
caffeine up to 2 cups/week
chemical less than weekly exposure

Moderate (M) = smoking 1-10 cigarettes/day
alcohol up to 6 times/week
marijuana 1-4 times/week
caffeine up to 6 cups/week
chemical less than weekly exposure with low precaution

High (H) = smoking more than 10 cigarettes/day
alcohol up to 3 times/day or more
marijuana greater than 4 times/week
caffeine a cup a day or more
chemical daily or almost daily exposure with or without precaution

The nondietary factors were chosen as potential variables because a few studies have shown that these substances affect drug metabolism by either inducing or inhibiting xenobiotic clearance

(59,61,64).

3. **Genetic Factors** Genetic factors were chosen as a potential variable affecting conjugated amino acid excretion since these are considered to be one of the important factors that makes xenobiotic metabolism of one individual differ from the other (64). In this study, genetic factors were assessed solely in terms of cancer incidences among family members.

Yes (Y) = had cancer incidences among the relatives

No (N) = had no cancer incidences among the relatives

C. The Use of Urinary Creatinine

Determination of urinary creatinine was also previously completed. The within and between subject variability in creatinine excretion (mg/kg body wt/ 24 hr) were determined for the population. Subjects whose creatinine excretion exceeded two standard deviations from the mean were excluded from further analysis.

In the course of the urinary amino acid conjugate study, the creatinine concentration(mg/ml) of each urine sample was used to determine an appropriate dilution factor for the urine samples and to express the concentration of conjugated compound in mmole/mmole creatinine/24 hr.

D. Determination of glycine and glutamine conjugates in urine

The method of Bidlingmeyer et al.(54) was used for the quantification of urinary conjugated glycine and glutamine. This method of amino acid analysis is based on the use of the Edman reagent, i.e., phenylisothiocyanate (PITC). PITC is used for the quantitative precolumn derivatization of free amino acids. PITC derivatization results in phenylthiocarbamyl (PTC) derivatives which then can be separated by High Performance Liquid Chromatography (HPLC). A general outline for this procedure is given in Figures 2 and 3.

Since the urinary excretion of peptides and compounds containing free amino groups other than conjugated amino acids is negligible in the daily excretion of urine in healthy adult population (30), the assumption was made that their contribution to measurements of urinary glycine and glutamine was not significant. Moreover, any substance, including peptides, which has a molecular weight greater than 10,000 was removed by ultrafiltration.

The quantification of the conjugated compounds was done by calculating the differences of amino acid before and after hydrolysis. Glycine conjugate, for instance, was determined by subtracting free prehydrolysate urinary glycine from total posthydrolysate glycine. For glutamine conjugate determination,

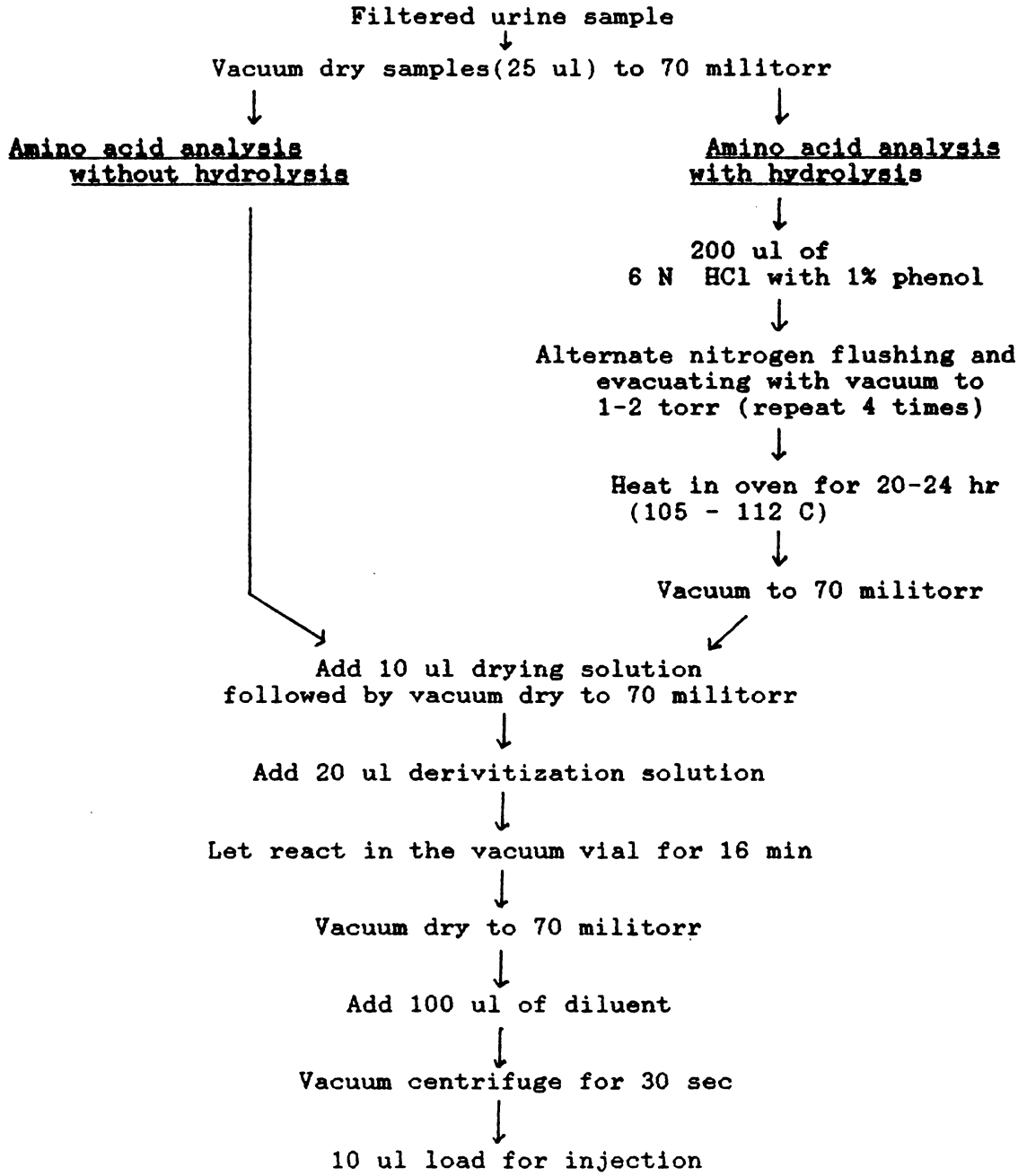


Figure 2. Analysis protocol

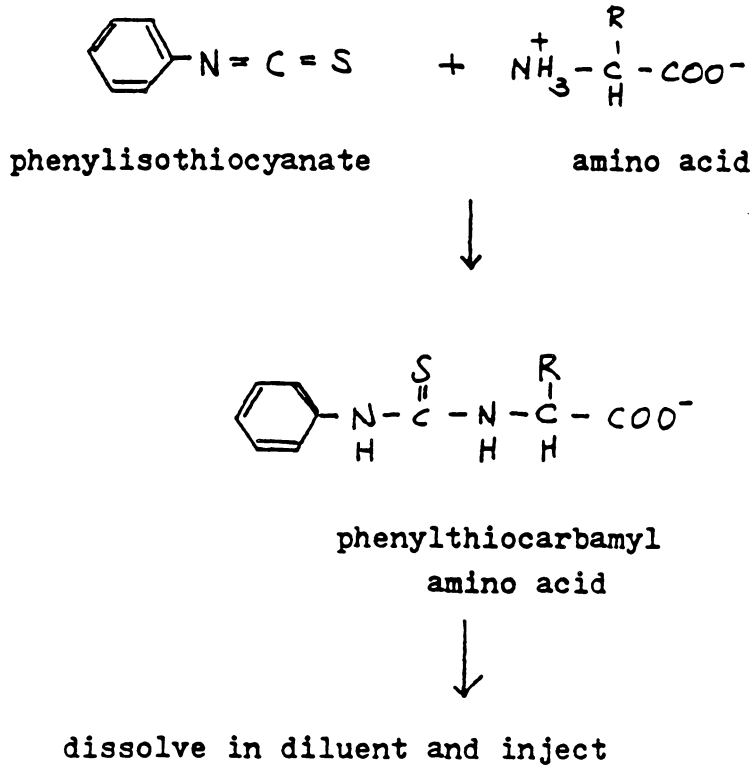


Figure 3. Production of phenylthiocarbamyl amino acids

free urinary glutamine and glutamic acid of prehydrolysate were subtracted from total glutamic acid, posthydrolysate since free glutamine converts to glutamic acid upon hydrolysis.

Before determining the urinary excretion of amino acid conjugates for the subjects in this study, an amino acid standard (500 pmol of each amino acid) and a urine sample spiked with 500 pmol of either hippuric acid or serylglutamine were analyzed. This preliminary study provided information as to whether peptide hydrolysis was complete. The results showed that 98% of glutamic acid was released from serylglutamine and 100% of glycine was released from hippuric acid. Furthermore, the results revealed that urine had no effect on hydrolysis.

The total time required for a complete analysis was approximately 50 hr, and the optimum number of samples per run was only six. Initially, results from one day urine samples of 117 subjects were to be used to determine interindividual (between subject) variation, whereas the results from a randomly selected subgroup of 40 subjects were to be used for intraindividual (within subject) variation. However, since a previous study of the urinary excretion of conjugated glucuronic acid (56) in the same subjects showed similar interindividual variation between the 117 samples and 40 subsamples, the urine of a randomly selected subgroup of 40 subjects were analyzed for determining both inter- and intraindividual variability of urinary conjugated amino acids.

The detailed procedure for urinary conjugated amino acid determination was as follows. A 1.0 ml aliquot of urine mixed with internal standard (methionine sulphone, 500 pmol) was filtered through a ultrafiltration device to remove urinary proteins greater than 10,000 daltons.[4] Any necessary sample dilution was done before ultrafiltration.

Filtered urine (25 ul) was placed in a 6 by 50 mm tube and dried under vacuum called PICO.TAG work station. After drying, the sample tube for the free amino acid analysis was placed in a dessicator and kept in a freezer until further analysis.

For hydrolyzation, 200 ul of 6 N HCl with 1 % phenol was added to the reaction vessel containing individual sample tubes (Figure 4). This allowed the use of HCl vapor for hydrolysis instead of HCl liquid. Removal of trace levels of oxygen from the hydrolysis reaction vessel was accomplished by alternate nitrogen flushing and vacuum evacuation. Then, the reaction vessel was placed in an oven (105 - 112°C) for 20 to 24 hours. Samples were dried again using vacuum.

The next step was the derivatization reaction. The method used a derivatization reagent which was prepared from a 1:1:1:7 ratio of PITC, TEA (triethylamine), water, and methanol. The reagent was prepared within 2 hr prior to addition. Adding 20 ul of reagent to the dried sample and sealing it in the vacuum vial for 20 minutes at room temperature resulted in the formation of the PTC amino acids. Prior to derivatization, 10 ul of drying

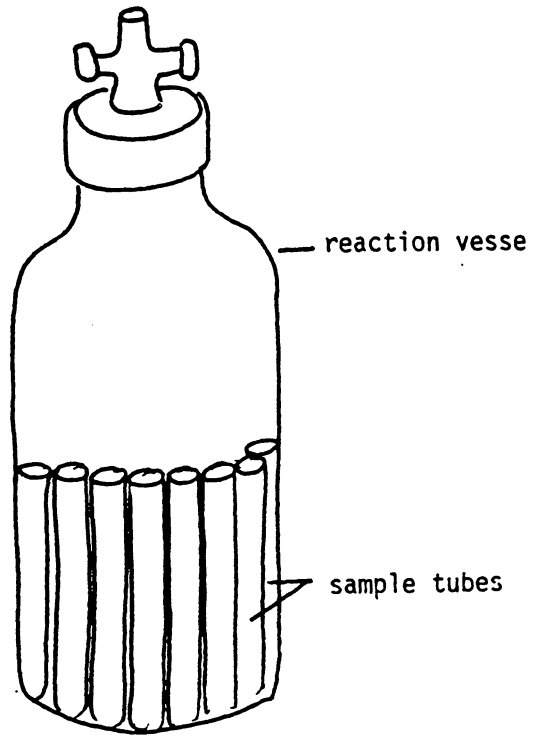


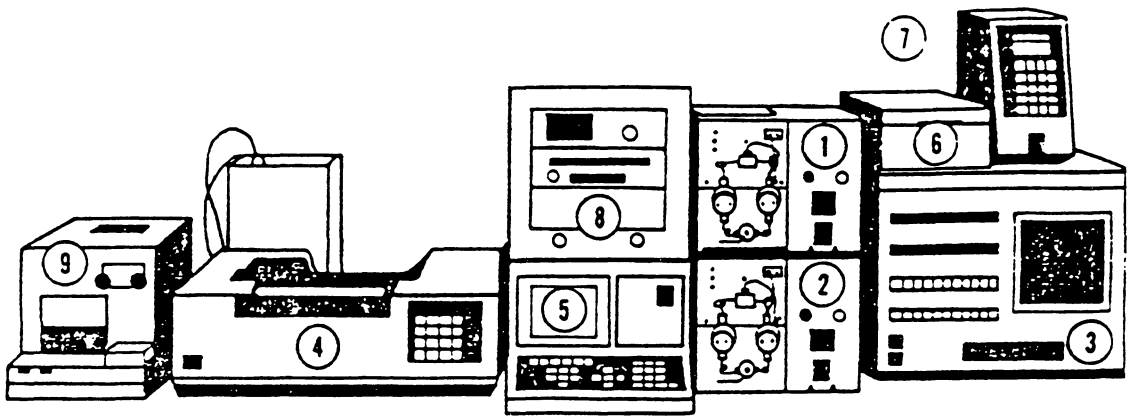
Figure 4. Sample tubes within reaction vessel

solution containing 1 M sodium acetate, methanol, and TEA (2:2:1) were added to each sample tube as a buffering reagent. The reagent was then removed using vacuum. When the sample was ready for injection, 100 ul of diluent (purchased from Water Association) was added to dissolve the dried sample.

The PICO.TAG chromatography system (Water Association) was used for the amino acid analysis (Figure 5). This chromatography system consists of two M510A solvent delivery systems and an M441 fixed wavelength detector (254 nm) controlled by a Waters 820 controller. The temperature is controlled within +/- 1° C with a column heater. Samples are injected in volumes of 10 ul using an M710B WISP(TM) auto injector. The columns are application specified PICO.TAG columns, packed in 13.25 by 0.75 inch hardware. It is quality controlled for rapid, high efficiency using reverse phase separation. The solvent system which consists of eluent 1 (buffer) and eluent 2 (acetonitrile, methanol, water) was kept under argon with an eluent stabilization system.

E. Data analysis

For the present study, a nonparametric statistical analysis, i.e. Kruskal-Wallis and Wilcoxon 2-Sample Tests were employed to test the hypothesis of differences between the respective variable groups. Additionally, Spearman Correlation Analysis was employed to determine the correlation between carbohydrate,



Component	Function
(1) Pump A	Delivery of eluent A
(2) Pump B	Delivery of eluent B
(3) Model 710B	Automated sample injection
(4) Model 730	Data reduction, plotting and documentation
(5) System Controller	Automated control of system (programming, methods, pumps)
(6) PICO•TAG Column	Performing the separation
(7) Column Heater/TCM	Maintaining column temperature
(8) Model 441	Detection of the derivatives
(9) PICO•TAG Work Station	Hydrolysis and pre-column derivatization of samples

Figure 7. PICO.TAG system (55)

protein, fat, total calorie intake and amino acid conjugate excretion. The results, however, do not confirm any treatment effect of variables on conjugated amino acid excretion since this study does not impose a measured quantity of any of the above variables on the subjects. Instead, it merely obtained intake or exposure frequency data regarding each variable according to the recollection of each subject.

For the purpose of the present study, a probability(P) value of between 0.1 and 0.2 was defined as marginally significant whereas a P value lower than 0.1 was considered statistically significant.

CHAPTER IV. RESULTS AND DISCUSSION

A. Subjects

The 40 subjects used in this study were derived from a group of 135 males, mean age 20.7 years. Dietary analysis indicated the subjects' food intake to be nutritionally adequate (66).

As mentioned earlier in Chapter III, Section C, three-day urinary creatinine excretions were measured and analyzed previously for between-subject and within-subject variability. Creatinine excretion remained relatively constant for an individual from day to day. Subjects whose creatinine excretions were at the extremes of the range (greater than two standard deviations from the mean which was 24.55 mg/kg of body weight/24 hr) and/or whose day-to-day creatinine excretions were inconsistent, were excluded from further analysis.

The mean, standard deviation and range for the urinary excretion of amino acids using three-day averages per 24 hr as shown in Table 2. The inter- and intra- subject variations of conjugated amino acid per 24 hr are shown in Table 3.

B. Dietary Factors

The mean and standard deviation for the urinary excretion of conjugated glutamine and glycine per 24 hr according to dietary variables are listed in Tables 4 and 5. Variables were class

Table 2. Urinary excretion of conjugated amino acids (n=40)

Conjugated amino acid	mean ± SD *	range
Glutamine		
mmole/24 hr	1.30 ± 0.66	0.28 -3.97
mmole $x10^{-2}$ /mmole creatinine/24 hr	8.74 ± 4.40	1.81 -20.77
Glycine		
mmole/24 hr	3.91 ± 2.08	0.70 -11.74
mmole $x10^{-2}$ /mmole creatinine/24 hr	26.38 ± 15.45	5.18 -87.94

* SD = Standard Deviation

Table 3. Inter and intra subject variation in urinary excretion of conjugated amino acid (n=40)

	Variation	
	inter CV *	Intra CV
Glutamine		
mmole/24hr	51.1	37.3
mmole/mmole creatinine/24 hr	50.3	36.0
Glycine		
mmole/24hr	53.4	31.4
mmole/mmole creatinine/24hr	58.6	32.8

* CV = coefficient of variation (%)

Table 4. Urinary excretion of conjugated amino acids according to dietary factors in mmole x 10 /mmole creatinine/24 hr

Parameter	Class	n	Glutamine	Glycine
			mmole x 10^{-2} /mmole creatinine/24 hr	
Vegetable	L	21	7.72 ± 2.31 * #	23.57 ± 9.34
	M	10	10.79 ± 5.55	34.71 ±21.32
	H	9	8.86 ± 2.61	23.69 ± 9.40
Fruit	L	29	7.96 ± 2.91 *	24.71 ±10.21
	M	7	11.62 ± 5.24	26.23 ±14.22
	H	4	9.36 ± 2.78	38.79 ±29.66
Meat	L	25	8.48 ± 3.04	29.49 ±15.48 *
	M	9	8.35 ± 4.02	22.85 ± 9.53
	H	6	10.40 ± 5.15	18.75 ± 7.59
Charbroil	L	32	8.42 ± 3.13	26.65 ±13.95
	M	0	-	-
	H	8	10.03 ± 5.11	25.30 ±14.25

* Marginally Significant (0.1<P<0.2)

Mean ± Standard Deviation

Table 5. Urinary excretion of conjugated amino acids according to dietary factors in mmole/24 hr

			Glutamine	Glycine
Parameter	Class	n	mmole/24 hr	
Vegetable	L	21	1.20 ±0.45 #	3.79 ±1.93
	M	10	1.38 ±0.62	4.37 ±2.04
	H	9	1.43 ±0.65	3.66 ±1.46
Fruit	L	29	1.27 ±0.56	3.96 ±1.82
	M	7	1.50 ±0.57	3.26 ±1.48
	H	4	1.19 ±0.28	4.68 ±2.68
Meat	L	25	1.20 ±0.45	4.20 ±2.10
	M	9	1.40 ±0.71	3.77 ±1.30
	H	6	1.56 ±0.59	2.90 ±1.04
Charbroil	L	32	1.26 ±0.53	3.93 ±1.86
	M	0	-	-
	H	8	1.46 ±0.57	3.81 ±1.89

Mean ± Standard Deviation

ified as low, moderate, or high intake of vegtables, fruit, meat and charbroiled food.

When expressed as mmole/mmole creatinine/24 hr (Table 4), excretion of glutamine conjugate showed a marginally significant difference ($0.1<P<0.2$) among groups of low, medium and high intake of vegetable and fruit. For glycine conjugate excretion, marginally significant differences were observed among low, medium and high intake groups of meat only. However, when values were expressed as mmole/24 hr (Table 5), none of the dietary factors showed statistically significant differences among each group for either glutamine or glycine conjugate excretion.

C. Non-Dietary Factors

The amino acid conjugates excreted per 24 hr of the sample for various environmental factors are given in Table 6 and 7.

Subjects were classified according to their use of tobacco, alcohol, marijuana, caffeine, and exposure to chemicals as being low, moderate, or high, and whether or not any incidence of cancer was present among their relatives.

When expressed as mmole/mmole creatinine/24 hr (Table 6), conjugated glutamine excretion for the factor alcohol showed marginally significant differences ($0.1<P<0.2$) and the factor chemical exposure showed significant difference ($P<0.05$) among low, medium, high groups. When expressed as mmole/24hr (Table 7),

Table 6. Urinary excretion of conjugated amino acids according to nondietary factors in mmole x 10 /mmole creatinine/24 hr

			Glutamine	Glycine
Parameter	Class	n	mmole x 10^{-2}/mmole creatinine/24 hr	
Tobaco	L	36	8.94 ± 3.61 #	26.46 ±14.51
	M	3	6.24 ± 3.62	24.14 ± 5.87
	H	1	9.17 ± 0	30.27 ± 0
Alcohol	L	31	8.22 ± 3.32 *	25.84 ±13.68
	M	9	10.54 ± 4.11	28.26 ±15.05
	H	0	-	-
Marijuana	L	35	8.99 ± 3.72	26.22 ±14.58
	M	2	8.24 ± 1.31	23.18 ±10.02
	H	3	6.20 ± 1.91	30.38 ± 5.96
Caffeine	L	23	9.01 ± 3.93	27.39 ±14.98 *
	M	5	7.52 ± 3.97	17.45 ± 8.35
	H	8	7.81 ± 2.88	30.70 ±15.12
Chemical	L	20	7.78 ± 3.87 **	25.61 ± 9.80 *
	M	10	11.19 ± 2.55	30.36 ±12.44
	H	10	8.21 ± 2.94	23.96 ±21.13
Cancer	Y	18	8.90 ± 4.63	30.68 ±16.93 *
	N	22	8.61 ± 2.55	22.87 ± 9.74

Mean ± Standard Deviation

* Marginally Significant ($0.1<P<0.2$)

** Significantly Different ($P<0.05$)

Table 7. Urinary excretion of conjugated amino acids according to nondietary factors in mmole/24 hr

Parameter	Class	n	Glutamine mmole/24 hr	Glycine mmole/24 hr
Tobacco	L	36	1.35 ±0.54 #	3.96 ±1.91
	M	3	0.86 ±0.39	3.56 ±1.23
	H	1	0.94 ±0.0	3.08 ±0.0
Alcohol	L	31	1.25 ±0.56 *	3.89 ±1.79
	M	9	1.47 ±0.42	3.97 ±2.13
	H	0	-	-
Marijuana	L	35	1.36 ±0.55 *	3.92 ±1.93 *
	M	2	0.92 ±0.02	2.58 ±0.72
	H	3	0.90 ±0.02	4.59 ±0.53
Caffeine	L	23	1.20 ±0.51	3.60 ±1.53 *
	M	5	1.18 ±0.61	2.76 ±1.34
	H	8	1.27 ±0.29	5.23 ±2.59
Chemical	L	20	1.19 ±0.54 *	4.07 ±1.97
	M	10	1.62 ±0.54	4.20 ±1.29
	H	10	1.20 ±0.43	3.28 ±2.07
Cancer	Y	18	1.23 ±0.54	4.34 ±2.21 *
	N	22	1.36 ±0.54	3.55 ±1.44

Mean + Standard Deviation

* Marginally Significant ($0.1<P<0.2$)

alchohol, chemical exposure, and marijuana showed marginally significant differences.

For conjugated glycine excretion (Table 6), when expressed as mmole/mmole creatinine/24 hr, marginally significant differences ($0.1<P<0.2$) were observed for the factors chemical exposure, caffeine, and cancer. When expressed as mmole/24 hr (Table 7), the factors of marijuana, caffeine, and cancer showed marginally significant differences, but chemical exposure did not show any differences among the levels.

The primary objective of this study was to quantify the urinary conjugated amino acids excretion, specifically glutamine and glycine conjugates, for a free living male population.

The mean urinary conjugated glycine and glutamine excretion for forty males in this study was lower than the mean values calculated from literature (Table 8). The upper range for glycine and glutamine conjugate values (Gly: 11.74 mmole/24 hr, Gln: 3.97 mmole/24 hr) for the forty males, however, did compare more closely to the values calculated from the literature.

Differences between the calculated literature values and those obtained from this study for amino acid conjugate excretion could be the result of several factors.

First, different analytical methods tend to give different measures of amino acids. The level of glutamine conjugates calculated from Woodson et al.(68) was obtained using a microbiological assay. This method determines amino acid

Table 8. Conjugated amino acids in the urine: comparison with the literature values

Literature	Subject (n)	Conjugate (mmole/24hr)	
		Glycine(range)	Glutamine(range)
Woodson(68) (1947)	8	- *	2.15 (0.49 -4.82)
Stein(18) (1953)	3	10.53 (10.00 -12.53)	3.59 (3.19 -4.35)
West(31) (1966)	-	9.53 (1.73 -17.33)	- *
This study	40	3.91 (0.70 -11.74)	1.30 (0.28 -3.97)

* Not reported

concentration by observing the specific growth requirement of a known organism which uses only that specific amino acid for its growth. However, Evered (25) has reported that the urinary free glutamic acid can be over estimated by microbiological methods, if decomposition or microbiological availability of combined glutamic acid, e.g., phenylacetyl glutamine, occurs. Higher conjugated glutamine value from the literature, therefore, may be the result of overestimation of glutamic acid, especially after hydrolysis. It is speculated that a similar problem might exist for glycine. Woodson et al.(68), however, did not report the level of glycine conjugates in their study. Furthermore, Woodson et al.(68) did not take free glutamine content into account when determining glutamine conjugates. Failure to subtract any free glutamine from total glutamic acid also may have resulted in an increased level of glutamine conjugates.

The more recent method, ion exchange chromatography by Stein (18), is unaffected by combined or homologous forms of amino acids. Therefore, it is more accurate within the limits of experimental error than the microbiological method. However, as with the microbiological method, Stein's method did not take free glutamine into account for glutamine conjugate determination which may have resulted in higher glutamine value.

Another possible reason for the differences between the experimental and the literature values may be due to sample selection and different sample sizes. It is possible that for a

larger population, as in the present experiment, the normal range in mmole per day of conjugated amino acid excretion is much greater and that the published data (calculated literature value) only contained subjects from the upper range. In this study most of the forty subjects excreted less than 9.0 mmole of conjugated glycine and 2.0 mmole of conjugated glutamine per day. However, 1 subject did excrete over 9.0 mmole of conjugated glycine and 6 subjects excreted over 2.0 mmole of conjugated glutamine per day.

A third possible reason for the lower amino acid values in this study, especially glutamine deals with potential losses during urine storage. Stein (18) noticed from his experiment that the large quantity of conjugated glutamic acid that was excreted in urine was present in some very labile combination. Therefore, even at 4°C, the labile form of conjugated glutamine may have escaped the determination of conjugated glutamic acid.

The secondary objective of this study was to determine the correlation between dietary/nondietary habits of subjects and their urinary conjugated amino acid excretion levels. The variables assessed along with a brief description of each can be found in Chapter III, Section B. To identify any significant differences in conjugated amino acid excretion at different levels of dietary and nondietary factors, nonparametric statistical analyses were performed. The Kruskal-Wallis Test was performed on the means for the dietary and nondietary factors except cancer and alcohol which were analyzed by the Wilcoxon 2-Sample

Test. These tests were used to compensate for the large variability and different sample sizes for each subgroup within a factor.

The results of this study showed no statistically significant differences among low, medium, high intake groups for dietary factors. When expressed in mmole/mmole creatinine/24 hr, only marginally significant ($0.1<P<0.2$) differences were observed for several dietary factors, i.e., vegetable, fruit and meat. However, because of the large variability and vastly different sample sizes for each group, no important conclusions can be drawn.

In agreement with results from the present study, Woodson et al.(68) demonstrated that the amount of all amino acids excreted by normal healthy human subjects showed no significant variations among individuals, although there was a wide variation in the type of "normal" diets, caloric intake, urine volume, and total nitrogen excretion. Eckhardt and Davidson (73) also reported that a large fluctuation in the type and quantity of protein ingested (varied from 50 gm of casein to an estimated 105 gm of mixed food protein) resulted in only small changes in the urinary excretion of free and combined amino acids. The study reported that the ratio of free to combined remained essentially constant for different protein intakes. This would indicate a proportional decrease in the excretion of amino acids in free and bound form thus maintaining the overall ratio. According to Stein et

al.(69) the quantities of hippuric acid and phenylacetyl glutamine observed in the urines from fasting individuals were similar to that of nonfasted normal adults which implies that these amino acid conjugates probably are normal metabolic products, and do not arise only as a result of the detoxication of dietary precursor.

As with the dietary factors, most of the nondietary factors examined showed marginally significant differences except for chemical exposure on glutamine conjugate excretion (mmole/mmole creatine/24 hr) which showed statistically significant differences. The factors that demonstrated a marginally significant difference on conjugated glutamine excretion were alcohol, marijuana, and chemical exposure whereas in conjugated glycine excretion, they were marijuana, caffeine and cancer.

The results obtained in this study are not without precedent. It has been known that dietary, environmental, and genetic factors influence xenobiotic metabolism. Gibson and Skett (64) have summarized the effects of various factors on drug metabolism as shown in Figure 6 and 7. As shown in Figure 8, protein and fat exhibit positive correlation which implies that increased consumption of these nutrients would increase drug metabolism. Furthermore, Kappas et al.(63) demonstrated a negative correlation exhibited by carbohydrate which implies decreased drug metabolism with increased carbohydrate consumption. In contrast, it is interesting to note that Spear-

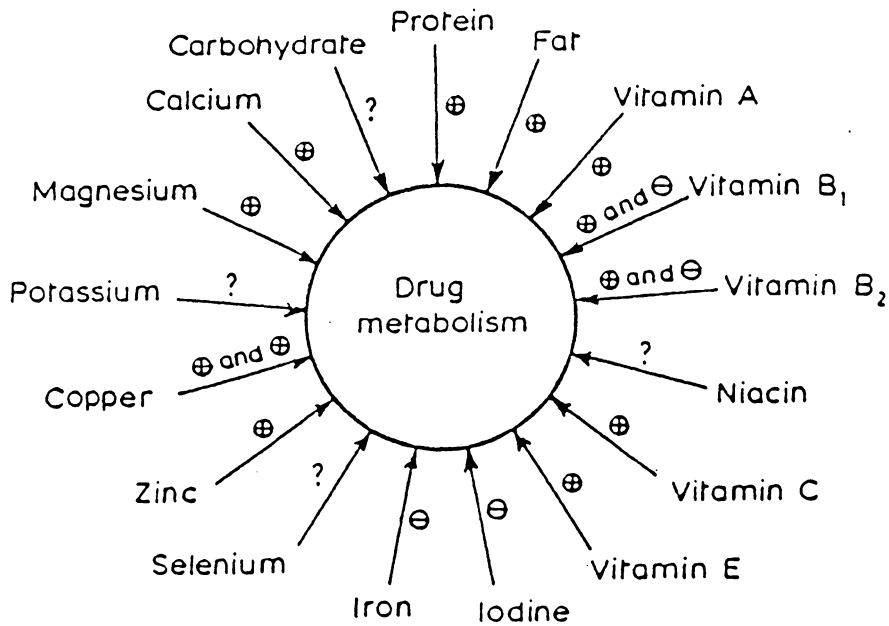


Figure 6 : Summary of the effects of dietary nutrients on drug metabolism (64).

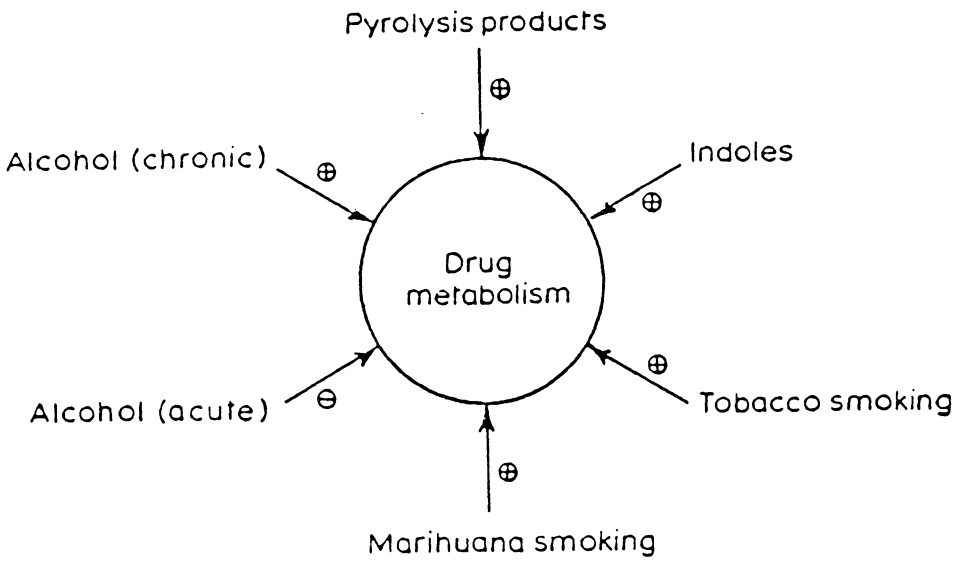


Figure 7 : Summary of the influence of dietary non-nutrients on drug metabolism (64) .

Table 9. Correlation between nutrient intake and urinary conjugated amino acids excretion

Nutrients	Spearman correlation coefficients	
	Glutamine	Glycine
Protein	0.12	-0.23 *
Carbohydrate	0.10	-0.01
Fat	0.07	-0.23 *
Kilocalories	0.13	-0.08

* Marginally significant ($0.1<P<0.2$)

man correlation analysis from the present study did reveal a marginally significant negative relationship between protein intake and conjugated glycine excretion. Fat intake also showed a marginally significant negative relationship with conjugated glycine (Table 9).

As mentioned in Chapter II, urinary amino acid conjugates excreted by normal adults consist primarily of hippuric acid and phenylacetylglutamine (26). Benzoic acid, which results in hippuric acid as a detoxication process, is present in many fruits and vegetables, especially in cranberries and prunes. Some food products such as ketchup are preserved with 0.1% of sodium benzoate thus contributing to the benzoic acid intake (31). Benzoic acid can also be formed by the oxidative breakdown of phenylalanine (22) and extensive aromatization of quinic acid by bacterial action in the intestine (70, 71). Since quinic acid is a component of tea, coffee, fruit and vegatables (70), it may be an important contributor to the normal output of hippuric acid. Nevertheless, the present study and several reports from other studies conclude that the relationship between the amount of food intake containing benzoic acid, phenylalanine or quinic acid and their contribution to glycine conjugate (hippuric acid) excretion was not significant.

The relative importance of internal (physiological) and external (environmental) factors in determining the human xenobiotic metabolism capacity is open to debate. Two different

perspectives can be considered. One view is that most of the differences in drug metabolism are due to internal factors, especially genetic differences, whereas the other indicates that differences are due to external factors including food, alcohol, and tobacco use (64). The influence of environmental factors, in addition to genetic differences, however, is very likely the cause of the interindividual variation seen in a population. Vessell (74) suggested that there are possibilities of dynamic interactions among dietary factors which further adds to the human drug metabolism along with genetic and environmental factors. He also explained that because of the interaction among these multiple factors, conflicting results obtained from drug clearance studies resulting in large interindividual variations are not necessarily contradictory.

In this study the three-day subject means were used to test significance. This was done to minimize the large interindividual variation, which most likely masks any effects of diet, environment or genetics upon urinary amino acid excretion. Unfortunately, the importance of these findings is still unclear due to the small and uneven sample sizes in the subgroupings. Also, the dietary information obtained from the food frequency questionnaire was to provide qualitative and not quantitative information. Therefore, the questionnaire did not provide a clear indication of actual dietary intake of the sample population. This study will, however, at least stimulate the

readers into further examination of one or more aspects of the amino acid conjugation of xenobiotics.

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

CHAPTER V. SUMMARY

The amount of urinary conjugated amino acids excreted by a free-living male population and the effect of certain factors, i.e., vegetable, fruit, meat, and charbroiled food intake, tobacco, alcohol, caffeine, and marijuana use, exposure to chemicals and familial cancer incidence were investigated. Three days of urine samples from 40 subjects who complied with the collection protocol were analyzed for each subject.

The mean conjugated glutamine excreted was 1.30 mmole/24 hr or 8.74×10^{-2} mmole/mmole creatinine/24hr. The mean values for conjugated glycine excreted was 3.91 mmole/24hr or 26.38×10^{-2} mmole/mmole creat/24hr.

For glutamine conjugate excretion, vegetable, fruit, alcohol, chemical exposure and marijuana use showed marginally significant differences among low, moderate and high consumers. For glycine conjugate excretion, meat, caffeine, chemical exposure, cancer and marijuana use showed marginally significant differences among low, moderate and high consumers.

An analysis of variance revealed a large degree of between-subject(inter) and within-subject(intra) variability. Glutamine and glycine coefficient of variations of intervariability were 51.1% and 53.4%, respectively, whereas intravariability of those were 37.3% and 31.4%. Probably, the large variability masked any effects of diet, environment or genetics upon the observed

urinary conjugated amino acid excretion. Therefore, better controlled research is necessary to further clarify the conjugated amino acid excretion pattern of a free-living population.

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APPENDIX

DATE _____
NUMBER _____

DETOXIFICATION PROFILE STUDY
1984
PRE-EXPERIMENTAL SURVEY

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

A. GENETICS

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

   a. Yes b. No

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

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

B. DIETARY

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

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

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

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

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

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

E. MEDICATIONS

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

   a. Yes b. No

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

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

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

   a. Yes b. No

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

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

F. ENVIRONMENTAL

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

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

EXAMPLE: ACETONE

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

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

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

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

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

   a. Yes b. No.

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

   a. Yes b. No

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

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

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

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

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

   _____ _____ _____
   _____ _____ _____

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

   a. Yes b. No

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

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

G. EXERCISE HABITS

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

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

2. If you answered Vigorous above, please describe:

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

   a. Yes b. No

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

   a. Yes b. No.

DATE _____
NUMBER _____

DETOXIFICATION PROFILE STUDY
1984
FOOD FREQUENCY QUESTIONNAIRE

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

D. OTHER SOCIAL DRUGS

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

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

4. During the past month, on the average, how many 6 oz. cups of hot/iced tea did you consume?

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

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

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

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

a. Yes b. No c. Uncertain

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

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

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

C. TOBACCO PRODUCTS

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

a. Yes b. No

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

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

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