THE URINARY EXCRETION OF SULFOCONJUGATES IN AN ADULT MALE POPULATION

by

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(ABSTRACT)

The amount of sulfoconjugates excreted by 135 free-living men and the effect of certain factors, i.e. familial cancer incidence, alcohol intake, tobacco smoking, marijuana smoking, medication and specifically analgesic use, exercise, protein intake and the number of meat and vegetable servings consumed daily, were investigated. One day of urine was analyzed for each subject and three days of urine were analyzed for a randomly selected sub-group of 40 subjects.

For the one-day sample, the average amount of bound sulfate excreted was 7.45 mmole/24 hr. (0.49 mole/mole creatinine). Total sulfate was 28.53 mmole/24 hr. (1.81 mole/mole creatinine). The values for the three-day sample were 7.65 mmole/24 hr. (0.49 mole/mole creatinine) and 28.92 mmole/24 hr (1.81 mole/mole creatinine) for bound sulfate and total sulfate, respectively.

Of the environmental factors under consideration, a significant difference was observed only for sulfate

excretion in relation to beer consumption. Beer consumers excreted more total sulfate in both one-day and three-day samples, and more bound sulfate in the three-day sample than non-beer consumers.

Analysis of the three-day data revealed intra- (within subject) and inter-individual (between subject) coefficients of variation of 57.42% and 90.03% for bound sulfate (mmole/24 hr). For total sulfate, intra- and inter-individual coefficients of variation were 22.76% and 67.35%, respectively.

The need to consider variation in experimental designs is discussed, and the necessity for further research regarding sulfoconjugate excretion and genetic and environmental influences is documented.

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INTRODUCTION

Animals are exposed to a variety of environmental and dietary compounds. Many of these compounds are nutritive and beneficial to the organism. They are either metabolised for their energy value or utilized as structural constituents. Other compounds are nonnutritive and may be exogenous or endogenous in origin.

Exogenous compounds usually gain entry into mammals via the gastrointestinal tract, lungs or skin (1, 2). These portals of entry are lined with a lipophilic membrane. Therefore, lipophilic compounds may passively diffuse through the membrane into the organism.

Many nutritive compounds such as amino acids, sugars and nucleic acids are not lipophilic. These compounds cross cell membranes via special transport systems. A nonnutritive hydrophilic compound may enter the animal by a compatible transport system. Consequently, even though most toxicants are absorbed by passive diffusion, several toxicants enter by facilitated transport. The effect of the compound's lipophilic character continues in the body because distribution is dependent on lipophilicity (2).

A series of metabolic pathways has developed in animals which increase the excretion rate of toxicants (1, 2, 3).

This metabolism is found primarily in the liver; however, other tissues such as the small intestinal mucosa, kidney and lungs also have the required enzymes (4). Williams in 1959 (3) divided the reactions into two broad classifications: Phase I and Phase II.

Phase I reactions are the biotransformation reactions; they are hydrolysis, reduction and oxidation. In Phase I a functional group is either added to or exposed on a toxicant. The altered toxicant in turn is more water-soluble and is a substrate for Phase II reactions.

Phase II reactions involve the conjugation of an endogenous compound to a toxicant, or more frequently, its derivative. The endogenous compound is highly polar, so the bound toxicant increases in hydrophilic character and is more readily excreted from the body. Excretion generally results from the inability of the polar conjugate to be reabsorbed in the kidney tubules or intestines (2).

Common conjugation agents are glucuronic acid, inorganic sulfate, glutathione and amino acids such as glycine and glutamine (5, 6). Phase II reactions require not only a conjugating agent but a high energy intermediate. With the exception of the amino acids and glutathione, the conjugating agent is activated and serves as the high energy intermediate.

One of the conjugating reactions, sulfation, has only been of moderate research interest until recent years. The lack of interest was probably due to the belief that sulfation was purely a detoxifying reaction. However, there is increasing evidence that sulfoconjugates may serve as the storage forms of hormones (7) or as reactive intermediates in carcinogenesis (8).

Sulfation in the human is relatively undetermined. Considering that sulfation plays an important biological role, it is surprising that little is known. Most research has focused on investigating the kinetics and degrees of sulfation of administered drugs by the isolation of corresponding sulfoconjugates (7, 9). Previous studies have been unable to indicate to what extent sulfation is used in humans, and therefore, have been limited in predictive value.

This study focused on the excretion pattern of all sulfoconjugates in a free-living male population. By studying a free-living population, the level of endogenous and exogenous sulfoconjugates was monitored and the effect of certain factors investigated. The factors considered were familial cancer incidence, alcohol intake, tobacco smoking, marijuana smoking, medication and specifically analgesic use, exercise, protein intake and the number of meat and vegetable servings consumed daily.

It is acknowledged that the multiple effects of a variable, i.e. cigarette smoking, affect all of the detoxication pathways. However, as an initial step in understanding the dynamic inter-relationship of organism and environment, this study focused on only one aspect of detoxication, that of sulfation.

Because most sulfation substrates are of a low molecular weight and the resultant sulfoconjugate is primarily excreted via the renal tubules (9), urine was collected. Urine collection also has the advantage of being a relatively noninvasive method of collection. The amount of sulfoconjugates excreted via urine in a 24-hour period was quantified for each subject. In addition, the intra- and inter-variability of sulfoconjugate excretion was assessed. Most methods regarding sulfate quantification are based on an indirect measurement (10). This study's protocol is no different; the sulfoconjugate concentration was obtained from the difference between the total sulfate and inorganic sulfate concentrations.

In summary, the objectives of this study are two-fold:

1) to determine the amount of sulfoconjugates excreted in a free-living male population.
2) to determine if certain factors, i.e. alcohol intake, tobacco smoking, etc., alter the sulfoconjugate excretion of the population.

REVIEW OF LITERATURE

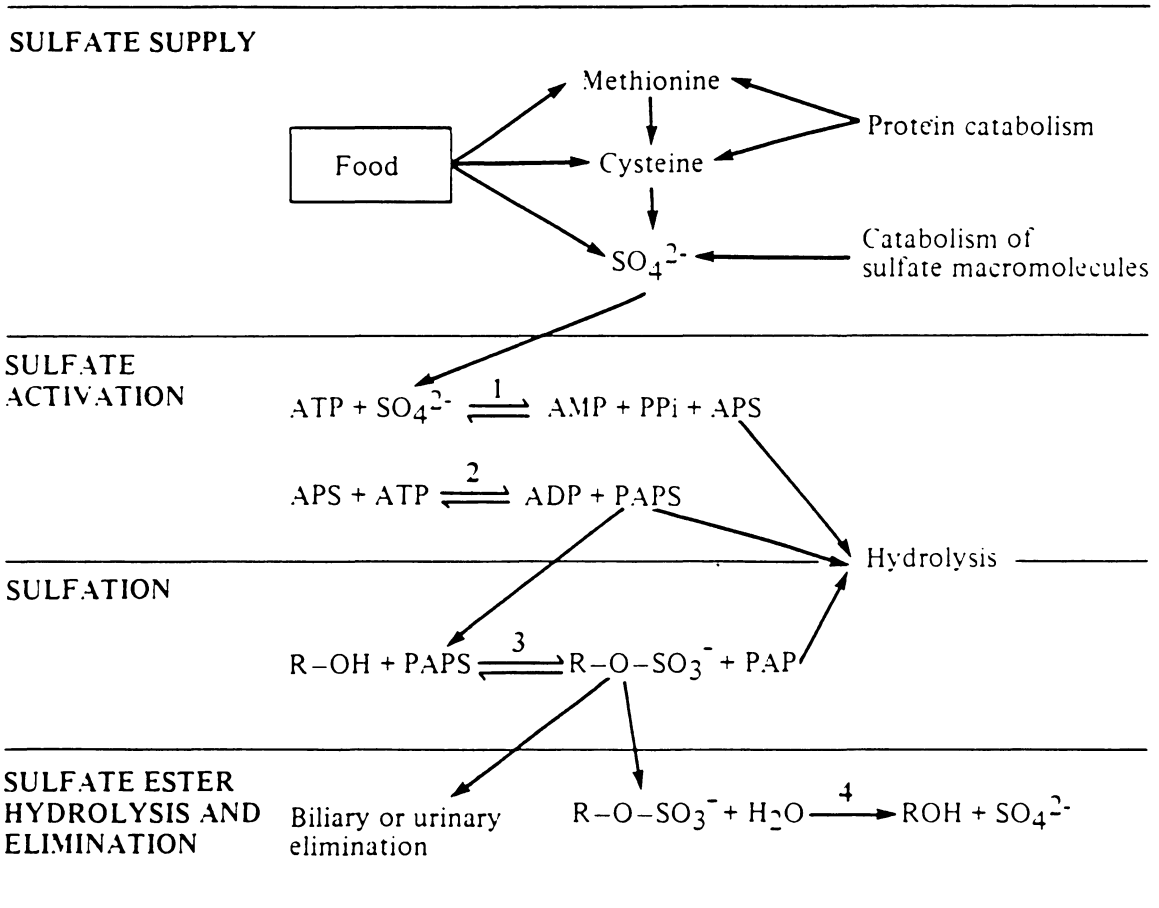
According to Mulder (11), sulfation was first reported by Baumann in the 1870's. Phenol sulfate was excreted when phenol was administered to patients. Baumann described the compounds as "ethereal sulfates;" however, these compounds are actually half-esters of sulfuric acid and are accurately referred to as sulfate esters.

Sulfation can be discussed in four broad areas: sulfate sources, activation, sulfation and elimination. Figure 1 depicts an overview of sulfation.

Sulfate Sources

The inorganic sulfate necessary for sulfation may be obtained from the diet or the catabolism of macromolecules. Dietary sulfate crosses the small intestine mucosal membrane via a carrier protein (12, 13). Exchanges such as sulfate-sulfate, sulfate-chloride, sulfate-hydroxyl ion and sulfate-sodium have been observed (13, 14, 15). Since the anion is almost quantitatively absorbed, it is fortunate that inorganic sulfate is relatively nontoxic.

Macromolecules such as glycosaminoglycans may also be a source of inorganic sulfate. Cysteine, released from the



ABBREVIATIONS:

SO_4^{2-}	inorganic sulfate
APS	adenosine 5'-phosphosulfate
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PAP	3'-phosphoadenosine phosphate

1. ATP-sulfurylase
2. APS kinase
3. Sulfotransferases
4. Sulfatases

Figure 1. Sulfate metabolism and sulfation.

[Adapted from (11)]

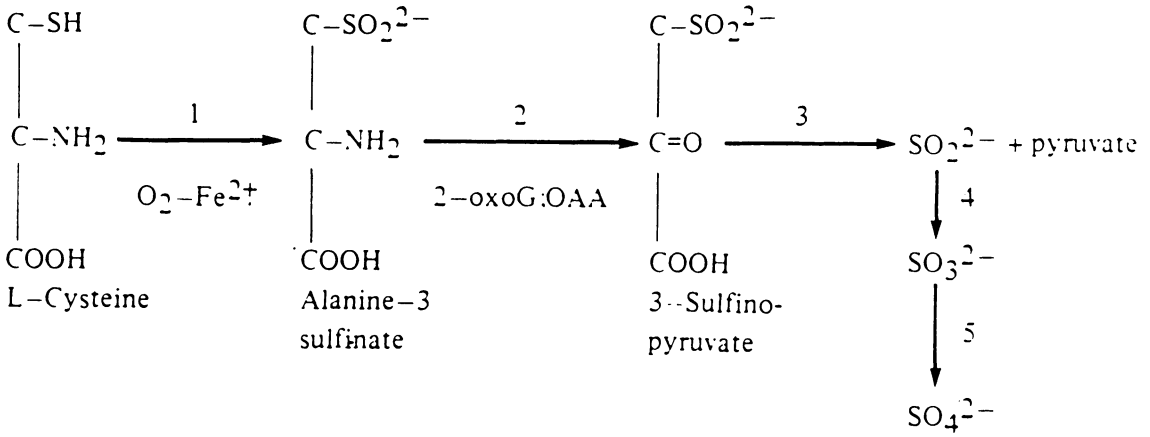
catabolism of these compounds and the oxidation of methionine, can be sulfoxidized with the eventual formation of inorganic sulfate and pyruvate (see Figure 2). It has been shown by previous studies that the amino acid and inorganic sulfate content of food is sufficient to meet the demands of sulfating all endogenous compounds and many exogenous compounds (12).

Activation

For sulfation to occur, the inorganic sulfate must first be activated to the high energy intermediate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) via the synthesis of adenosine 5'-phosphosulfate (APS) (16). Figure 3 contains the structures of APS and PAPS.

ATP-sulfurylase which catalyzes the first reaction is specific for adenosine triphosphate (ATP) and inorganic sulfate. One enzyme is capable of binding several moles of ATP. APS-kinase which converts APS to PAPS has a high affinity for APS, but it is not specific for ATP. The reaction this enzyme catalyzes is irreversible due to its very exergonic nature.

Both APS and PAPS may be degraded by nucleotidases or sulfohydrolases. The breakdown of APS appears to be low because APS-kinase quickly converts APS to PAPS. However, the amount of PAPS breakdown is unknown.



ABBREVIATIONS

2-oxoG 2-oxoglutarate
OAA oxaloacetate

1. Cysteine dioxygenase
2. Cysteine sulfinate transaminase
3. non-enzymatic, rapid step
4. non-enzymatic, rapid step
5. Sulfite oxidase

Figure 2. Sulfoxidation of cysteine to inorganic sulfate.

[Adapted from (12)]

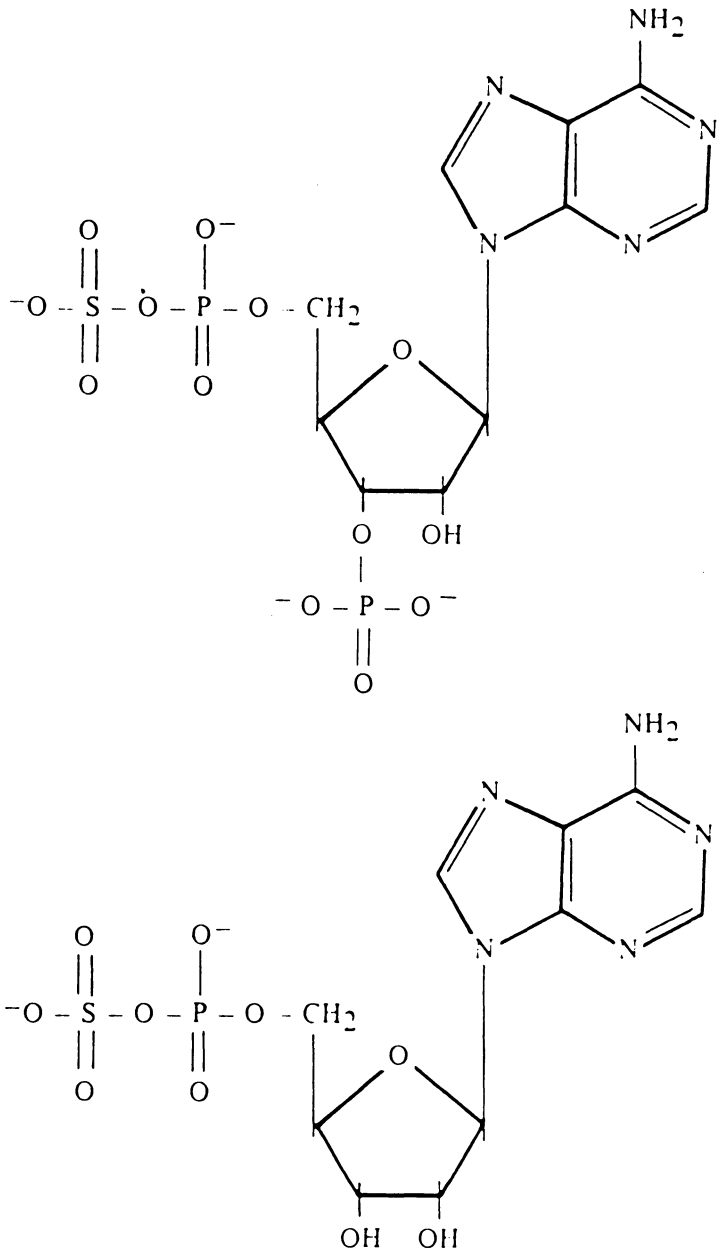


Figure 3. Chemical structure of 3'-phosphoadenosine 5'-phosphosulfate (top) and adenosine 5'-phosphosulfate (bottom).

[Adapted from (16)]

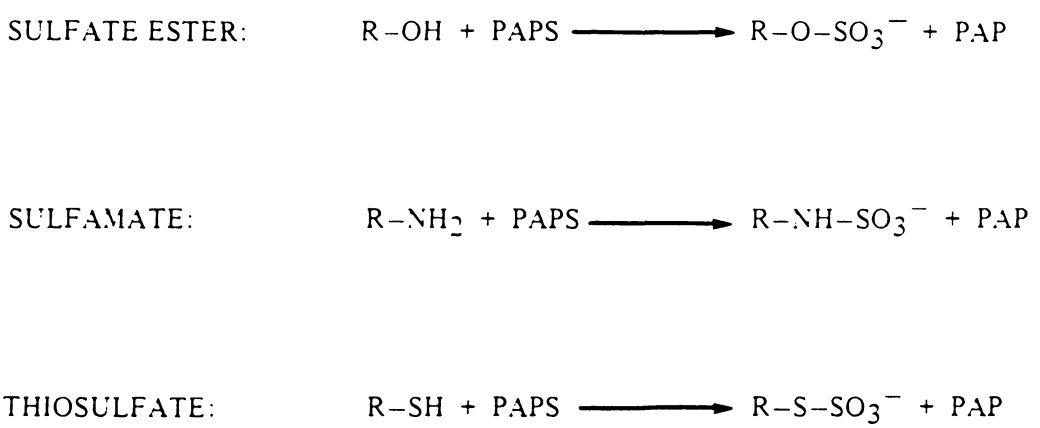
Sulfation and Sulfation Products

Sulfation is the actual transference of activated sulfate to an appropriate substrate (5, 17). Phenolic compounds are the most common type of substrate. Aliphatic alcohols, aromatic amines and thiol-containing compounds are also sulfated but to a lessor extent (5).

Sulfation is catalyzed by a group of enzymes called sulfotransferases (17). The enzymes are found in the cytosol, although, they may be bound to the endoplasmic reticulum. Currently, it is not known if there are a few relatively nonspecific enzymes rather than many highly specific enzymes.

Reactions catalyzed by sulfotransferases are usually irreversible (see Figure 4). As previously stated, the formation of sulfate esters occurs readily. On the other hand, thiosulfates of endogenous compounds do not occur in appreciable amounts in animals. To date no xenobiotic thiosulfate has been isolated (18).

Sulfate esters are usually found as salts. They are hydrolyzed more readily in acidic than alkaline or neutral solutions. The oxygen-sulfur bond is split, which generates the parent hydroxy compound, or sometimes a derivative. Aryl sulfates hydrolyze faster than alkyl



ABBREVIATIONS

PAPS 3'-phosphoadenosine 5'-phosphosulfate

PAP 3'-phosphoadenosine phosphate

Figure 4. General reactions of sulfoconjugate formation.

[Adapted from (1)]

sulfates due to the electron-withdrawing substituents in the aromatic ring.

Sulfamates, like sulfate esters, are usually found as salts. The nitrogen-sulfur bond is very labile in acid but stable in alkaline solutions. Similar to the sulfate esters, the aryl sulfamates hydrolyze more readily than the alkyl sulfamates.

Any xenobiotic with a phenolic, hydroxy or amine group may be sulfated in vivo. Endogenous compounds that are sulfated include lipids, bile salts, steroid hormones and neurotransmitters (7, 19, 20).

Elimination

The mechanisms of biliary and urinary excretion are unknown; yet, several factors have been elucidated. For example, the presence of a polar group such as a sulfate moiety definitely diminishes reabsorption of the conjugate; thus, elimination occurs. Many conjugates are bound to plasma proteins (21); bound conjugates are too large to pass through hepatic membranes or glomerular filtration and undergo elimination. Therefore, the strength of the plasma protein-conjugate bond also affects the amount of conjugate excreted.

Overall, biliary and urinary excretion complement each other. In the rat, if a compound has a molecular weight

less than 350, then the compound is excreted in the urine. A molecular weight over 450 leads to biliary excretion while compounds with molecular weights between 350 and 450 may be excreted in the bile or urine (22).

Hydrolysis

Sulfoconjugates are generally eliminated from the body. Yet, if the sulfate moiety is cleaved off before the compound can be excreted, the compound may stay within the organism. The enzymes which hydrolyze sulfoconjugates are known as sulfatases.

Sulfatases specifically catalyze the hydrolysis of sulfate esters. The activity of the enzymes on three synthetic aryl sulfates have enabled researchers to categorize the enzymes into two classes. Type I sulfatases are localized in the microsomal fraction. The substrates for these enzymes are steroid sulfates. Type II sulfatases are lysosomal enzymes. The turnover of macromolecules such as cerebroside sulfate and glycosaminoglycan is catalyzed by these enzymes (9).

Other lysosomal enzymes include sulfamatases and sulfatophosphate sulfatases. Sulfamatases hydrolyze nitrogen-sulfate linkages which frequently occur in connective tissue compounds such as heparin (9). As the enzyme's name implies, the sulfatophosphate moiety of APS

and PAPS is cleaved off by a sulfatophosphate sulfatase. As previously stated, the frequency of nucleotide degradation is unknown.

The aforementioned enzymes will hydrolyze any suitable substrate. This fact is illustrated by the activity of the sulfatases on the synthetic aryl sulfates.

The Fate of Sulfoconjugates

The main purpose of sulfation is detoxication. Consequently, once a compound is sulfated, it is generally excreted, either in the bile or urine. In fact, many administered sulfate esters have been recovered in the urine unchanged (9). Thus, sulfoconjugates appear to be the end products of drug metabolism.

On the other hand, sulfoconjugates of endogenous compounds may be metabolic intermediates of the active form of the respective compound. For example, L-tyrosine sulfate is an integral component of mammalian fibrinogen (9). In addition there is considerable speculation concerning the steroid sulfates as "storage" forms for various hormones (20). Hormonal activity, then, would occur when a sulfatase cleaved the sulfate moiety off the sulfoconjugate.

The fate of a sulfoconjugate depends greatly on the site of sulfation (23). As previously stated, a compound

sulfated in the kidney is often excreted via the urine. In the liver, however, the conjugate may be eliminated in the bile or it may enter the blood to be distributed. The distribution may lead to urinary excretion or further metabolism in another tissue. Even with biliary excretion, the conjugate may undergo metabolism by the intestinal microflora perhaps leading to enterohepatic circulation for the toxicant.

Availability of Inorganic Sulfate

Sulfation, whether in the liver or in another tissue, depends on the availability of inorganic sulfate in the tissue. Mulder and Scholtens (24) observed the rapid incorporation of [^{35}S]-sulfate into harmol by measuring the biliary excretion of harmol sulfate. A tracer dose of [^{35}S]-labeled sodium sulfate and harmol were simultaneously administered intravenously. After one minute, harmol-[^{35}S]-sulfate appeared in the bile. The delay of one minute is attributed to the dead space of the bile ducts.

Apparently the plasma sulfate concentration had rapidly equilibrated with the liver sulfate concentration. This conclusion is supported because the radioactivity of harmol sulfate decreased as the plasma radioactivity decreased. Confirmation of [^{35}S]-sulfate's rapid equilibration with

endogenous sulfate came from experiments utilizing perfused rat liver (25) and isolated hepatocytes (26). It would seem, then, that the plasma sulfate concentration is an accurate measurement of the direct sulfate availability in the liver.

Several researchers have administered high doses of a drug in humans and observed a decline in sulfation (7). Since the subsequent administration of a sulfate source increased sulfation, they concluded that the inorganic sulfate pool had been depleted. If the human serum concentration of inorganic sulfate is considered to be 0.3mM (27, 28, 29, see Table 1) with a distribution volume of 20% body weight, then the administered doses would not have been high enough to utilize all of the immediately available sulfate. Unfortunately, plasma sulfate was not measured in these experiments. The increase in sulfation upon administration of a sulfate source may have been due to an enhanced sulfation rate rather than restoration of inorganic sulfate to physiological levels.

The relationship between plasma sulfate concentration and sulfation was further explored by Krijgsheld et al. (27). They fed rats a low protein diet for four days before administering a constant infusion of harmol to the bile duct-ligated animals. Sodium sulfate was then added to the infused solution in increasing concentrations at

Table 1.

Pool Size of Inorganic Sulfate in Various Species

Species	Serum sulfate (mM)	Sulfate distribution volume (% of body wt.)	Directly available sulfate (umol/kg)	Urinary excretion of sulfate (umole/hr/kg)
Man	0.3	20	60	15
Rat	0.9	25	225	70
Mouse	1.2	25	300	--
Dog	1.4	20	280	25
Cow	1.8	25	450	--
Rabbit	2.0	25	500	25

Adapted from (27).

regular intervals. Initially, the plasma inorganic sulfate and urinary harmol sulfate concentrations were low; however, as the plasma level of inorganic sulfate increased upon the addition of sodium sulfate, the excretion of the sulfoconjugate also increased with a concomittant decrease of harmol glucuronide excretion.

It is interesting to note that plasma sulfate levels higher than physiological levels did not significantly increase harmol sulfate excretion in this experiment. It appears that drugs such as harmol have a low sulfation Km relative to the plasma inorganic sulfate concentration in the rat. Thus, the plasma level would have to be severely reduced before the sulfation rate is altered. However, the sulfation Km for other drugs is similar to the plasma inorganic sulfate concentration (7, 25, 30). Any change in the plasma concentration would, therefore, affect the sulfation rate for those drugs.

Undeniably, sulfate depletion can occur; but the sulfate pool seems to be large enough to handle a range of drug doses. Since sulfation is considered to be an important detoxication pathway (3, 5, 6), it is inconsistent for the sulfate pool to be readily depleted. In addition, a sulfate deficiency would result in the impaired function of a number of endogenous compounds (7, 19, 20).

Research regarding inorganic sulfate availability is difficult to summarize because the studies generally differ in the type and dose of drug administered. Furthermore, most studies were conducted with a single dose. Consequently, a large dose may not indicate sulfate depletion but a metabolic shift toward glucuronidation, sulfation's counterpart (7).

Competition with Other Detoxication Pathways

Most research concerned with sulfation's relationship with the other detoxication pathways has focused on the competition between sulfation and glucuronidation. As previously stated, glucuronidation is enhanced as sulfation decreases. One factor which may influence the glucuronidation:sulfation ratio for a cell is the amount of substrate in that cell. While glucuronidation has a low affinity and high capacity for most compounds, sulfation has a high affinity and a low capacity. Therefore, as the substrate concentration in the cell increases, sulfation becomes saturated and the excess substrate is shifted toward glucuronidation. Factors such as "cosubstrate availability, lipid solubility of the substrate, substrate specificity of the transferases, pharmacokinetic factors, and changes in uptake by tissues as the dose increases (31)" also affect the glucuronidation: sulfation ratio.

Sulfation's interaction with glutathione and the amino acid detoxication pathways is relatively unknown. However, sulfation of toluene and xylene as an intermediate for mercapturic acid formation has been demonstrated (32). In addition, since glutathione and inorganic sulfate share a common precursor, cysteine, an increase in glutathione turnover may decrease the availability of inorganic sulfate, and vice versa (33). Furthermore, research indicates that glutathione is not normally converted to inorganic sulfate, via cysteine, when sulfate depletion occurs.

Species Differences

Species differences for sulfation have been studied primarily using phenol (7). Results indicate that most animals can sulfate, but the species differ widely in the glucuronidation:sulfation ratio (see Table 2). For example, research with a number of drugs indicate the feline's deficient capacity for glucuronidation; consequently, sulfation is preferred. On the other hand, the pig seems to be deficient in sulfation. Furthermore, studies of common laboratory animals show that while the rat and mouse readily excrete both sulfoconjugates and glucuronides, the rabbit and guinea pig prefer glucuronidation. These findings should be regarded with

Table 2.

Conjugate Excretion of Orally Administered Phenol in Various Species[a]

Species (n, sex)	Dose (umol/kg)	Radioactivity excreted in 24-hr. urine (% of dose)	Phenyl sulfate (%)	Phenyl glucuronide (%)
Primates				
Man (3,m)	0.11	90	77	16
Rhesus monkey (2,f)	530	43	65	35
Squirrel monkey (3,f)	266	31	7	58
Carnivores				
Ferret (3,f)	266	51	28	40
Cat (3,f)	266	59	87	--[b]
Lion (3,f)	108	77	99	--
Dog (1,f;1,m)	266	58	50	18
Hyena (1,f;1,m)	108	31	90	--
Miscellaneous				
Elephant (1,f)	108	49	73	25
Pig (3,f)	226	51	--	100
Hedgehog (2,m)	213	38	75	15
Fruitbat (2,f)	266	54	10	90
Rabbit (3,f)	266	48	45	46
Chicken (3,f)	266	57	78	22
Rodents				
Rat, Wistar (3,f)	266	95	54	42
Mouse, ICI (3x10,f)	266	66	46	35
Gerbil (2,f)	266	55	42	35
Hamster (2,f)	266	75	26	43
Guinea pig (2,f)	266	64	18	88

[a] When the percentages in the last two columns add up to less than 100%, the remainder consisted of other metabolites, such as quinol conjugates.

[b] Means not detectable.

Adapted from (7).

caution, though, due to the single-dose design of the studies. As stated earlier, the glucuronidation:sulfation ratio is dose-dependent.

Extrahepatic Sulfation

In addition to the effect of dose concentration and frequency, the amount of conjugate excreted is influenced by extrahepatic sulfation. The difference in dopamine metabolism of the dog as opposed to that of the rat and guinea pig was traced to the intestinal mucosa's ability for first-pass sulfation (34). First-pass sulfation is the sulfation of compounds during their absorption in the mucosal cells or their passage through the liver.

In some animals only the liver of the tissues studied was found to sulfate while other animals formed more sulfoconjugates of administered drugs in the lung, kidney and small intestines than in the liver (35). Even though the tissues just mentioned have been primarily studied, it seems that most organs can sulfate as indicated by the wide distribution of sulfotransferases (7). The importance of extrahepatic sulfation is unknown at this time. Factors determining in which tissue a substrate is sulfated are also unknown; however, substrate structure is a likely factor (7).

Of the extrahepatic tissues, the intestinal mucosa may have an important role in drug metabolism. For example, intravenous administration of isoprenaline in man and dog resulted in the drug being excreted unchanged in the urine. However, the sulfoconjugate of isoprenaline was the main excretion form when the drug was administered orally. Further research showed isoprenaline sulfation occurred in the intestine (7).

Age and Sex Differences

Little is known regarding the effects of age and sex on sulfation. There has been no research on the elderly. On the opposite end of the chronological scale, sulfation in fetal laboratory animals and fetal humans has been detected, sometimes at levels greater than that which occurs in adults (7). Since sulfation plays a role in growth and regulation, it is important that the pathway occurs in fetal animals. Fortunately, inorganic sulfate readily passes through the placenta (12).

As for sex differences, the female rat seems to have a slower sulfation rate than the male rat. As may be expected, the female rat has an increased glucuronidation rate as compared to the male (7). Sex differences have not been observed in other animals.

Methods Regarding the Quantification of Sulfoconjugates

Historically, methods concerned with the quantification of all sulfoconjugates in a sample have relied on an indirect measurement; the difference between hydrolyzed and non-hydrolyzed measurements for the same sample represents the concentration of sulfoconjugates in that sample (10). Consequently, an important part of any such protocol is the quantification of inorganic sulfate, either naturally found in the free form or released from a sulfoconjugate. Inorganic sulfate is a difficult compound to quantify; it has no chromogenic properties and only a few reagents can precipitate the anion, most notably barium and benzidine. Most methods, therefore, are based on a barium or benzidine precipitate when determining urinary inorganic sulfate concentrations. Since benzidine is carcinogenic, this study considered only the methods concerned with barium precipitation.

Folin (36) was the first person to perform a definitive study on the formation and characteristics of barium sulfate precipitates. Since that time, numerous methods were developed to quantify urinary inorganic sulfates via a barium sulfate precipitation (10). Gravimetric (36), turbidimetric (37, 38) and flame spectrophotometric methods (39, 40; 41) have been or currently are commonly used. Gravimetric analyses offered a precise but laborious

protocol. Turbidimetry and flame spectrophotometry are faster methods; unfortunately, they also may be less precise since a rapid precipitation may trap unwanted material (contaminants) in the precipitate. However, recoveries of spiked samples have generally been 90% or higher, indicating turbidimetry and flame spectrophotometry are acceptable methods.

Accurate turbidimetric measurements depend on the even distribution of barium sulfate throughout a colloidal suspension (10). Since several experimental factors, i.e. pH, rate of precipitation, mixing conditions, etc., affect turbidity, a very rigid protocol must be developed and followed. Consequently, methods utilizing the atomic absorption spectrophotometer were considered due to the methods' reduced sensitivity to these experimental conditions.

Roe et al. (39) used the atomic absorption spectrophotometer in quantifying sulfoconjugates. They were estimating the amount of sulfur in urine and feces by oxidizing the sulfur in sulfur-containing compounds to a sulfate moiety. The products were hydrolyzed with the freed sulfate precipitated, picked up in a solution of disodium EDTA and measured by the atomic absorption spectrophotometer. Hydrochloric acid was used as the hydrolyzing reagent.

In another study, Lloyd et al. (41) compared hydrochloric and nitric acids in hydrolysis efficacy of carbohydrate sulfate. Recoveries with 6N hydrochloric acid for 12-24 hours at 100 ^{o}C ranged from 75% to 88%. Apparently certain carbohydrate sulfates are resistant to hydrochloric acid hydrolysis. Unfortunately, higher concentrations of this acid will char the sample and lead to low sulfate determinations (36, 41).

Fuming nitric acid for 24 hours at 100 ^{o}C produced a recovery range of 97% to 104%. Consequently, Lloyd et al. recommended the use of nitric acid as a nonspecific hydrolysis reagent of sulfoconjugates. Because of their detailed explanation and excellent recovery of various carbohydrate sulfoconjugates, Lloyd et al.'s (41) protocol was selected for this study.

Summary

Sulfation is a metabolic pathway which occurs in most animals. The liver is the primary site of sulfation but tissues such as intestinal mucosa, lung and kidney also contain the required enzymes. At this time, the importance of extrahepatic sulfation is undetermined.

In brief, sulfation is the transferance of activated sulfate to an appropriate substrate, usually phenolic compounds. The resultant sulfoconjugate may be excreted,

stored, as in the case of certain steroid sulfates, or serve in a structural or metabolic capacity. If hydrolysis via sulfatase activity occurs, the released sulfate ion may be utilized again in the sulfation pathway and the substrate may be free for metabolic activity. In the case of xenobiotic metabolism, sulfation is almost invariably a detoxifying reaction; therefore, the sulfoconjugate form is desired for its enhanced excretion of the xenobiotic.

In vivo, sulfation competes with other detoxication pathways. Most research has focused on sulfation's interaction with glucuronidation. As the dose of a drug increases, the primary detoxication pathway shifts from sulfation to glucuronidation. If the dose is sufficiently large, the shift in pathways may be due to a depletion of activated sulfate. However, research indicates that an increase in the glucuronidation:sulfation ratio occurs before the availability of inorganic sulfate is severely restricted. Consequently, other factors such as co-substrate availability and enzymatic substrate specificity must be considered in evaluating sulfation's contribution to detoxification.

Research has primarily focused on the kinetics of and conditions surrounding the sulfation of administered drugs. A few studies have been published which describe methods to quantify sulfoconjugates in general.

Unfortunately, these studies were conducted with few human subjects, if any, and the data were not discussed in relation to excretion pattern or influencing factors. As a result the studies are limited in predictive value. This study will investigate the amount of sulfoconjugates humans excrete under "normal" conditions and to assess several factors as to their possible effects on the amount excreted.

MATERIALS AND METHODS

Subjects

A voluntary group of men from the Blacksburg community were the subjects for this study. Posters displayed throughout the Virginia Tech campus and the town of Blacksburg, as well as advertisements in the campus newspaper, were used to recruit subjects.

One hundred sixty-six subjects were accepted without bias on the conditions that they were not under a physician's care and were willing to maintain a constant weight during the urine collection period. The subjects were required to complete three forms: consent form (approved by the Chairman of the Internal Review Board for Human Research at Virginia Tech), and environment exposure and food frequency questionnaires (Appendices A, B, C). Confidentiality was maintained by assigning a number to each subject which appeared on the questionnaires and urine bottles in place of a name.

Urine Collection

Each subject collected his urine for three consecutive 24-hour periods. Due to the limited number of collection bottles, the subjects were assigned to one of four groups.

Each group, composed of approximately 40 subjects, collected urine for one week. All collections were made on Tuesday, Wednesday, and Thursday. Samples were brought to the lab at the end of each 24-hour period. Each 24-hour period began with the second voiding of urine and continued to and included the first voiding of the next day. Subjects were offered a cash payment of $10.00 as compensation when they completed the three-day collection.

Each subject was provided with as many one-liter polypropylene bottles as he needed in one 24-hour period. Each bottle was carefully acid washed and autoclaved to prevent microbial growth. Bottles were then wrapped in aluminum foil and placed in a brown paper bag to minimize potential photo-oxidation of the conjugates before being given to a subject.

Subjects received instructions for the urine collection in written and verbal forms one day before the collection began (Monday). The subject's height and weight were recorded the same day. On the following Wednesday and Thursday, the weight of each subject was recorded again.

Urine Handling

Each day the urine bottles were returned to the laboratory, the urine for a subject was mixed, and the

total volume and pH recorded. A 200ml aliquot was placed in a labeled 250ml polyethylene bottle and frozen at -20°C. Approximately 50ml of urine was stored in a labeled bottle and frozen for creatinine analysis.

In preparation for creatinine or sulfate analysis, samples were moved from the freezer to the refrigerator approximately 24 hours before the start of the analytical procedure. The sample's temperature was raised to room temperature by placing the sample in warm water a few minutes before the sample was used.

Inorganic Sulfate Analysis

Quantification of inorganic sulfate was determined by modifying Lloyd et al.'s protocol (41). Duplicate 1ml aliquots of each sample were pipetted into polypropylene tubes (Sarstedt 17ml with snap-on caps). Three ml de-ionized water, 5ml barium chloride (1.78% w/v aqueous $BaCl_2$) and 0.18ml concentrated hydrochloric acid were added to each tube. Samples were centrifuged cold (9°C) at 4500 rpm for 30 minutes. The supernatant was discarded and the pellet washed twice with 5ml de-ionized water. Samples were centrifuged again and the supernatant discarded after each washing. The pellet was dissolved in ethylene diaminetetra-acetate solution (10gm EDTA, free acid form,

dissolved in 100ml concentrated ammonium hydroxide containing 19.07g potassium chloride, and diluted to a final volume of 1000ml with de-ionized water) to bring the volume to 5ml. A 0.2ml aliquot was then diluted to 10ml with the EDTA solution before reading on the atomic absorption spectrophotometer.

A Perkin Elmer Atomic Absorption Spectrophotometer Model 3030 was fitted with a barium hollow-cathode tube (L233, L733, L1788 Series). The equipment was operated at recommended manufacturer specifications. The atomic absorption spectrophotometer was "zeroed" to the EDTA solution. Varying concentrations of sodium sulfate treated as described above were used to delineate the standard curve. Treated urine samples were aspirated into the flame of the atomic absorption spectrophotometer. Concentrations were determined from the standard curve and corrected for the dilution factor. If the percent error between duplicates was greater than 5%, then the respective sample was analyzed again.

Sulfoconjugate Analysis

Quantification of sulfoconjugates began by freeze drying duplicate 1ml aliquots of urine. A dried sample was subjected to 1.5ml fuming nitric acid at 100 ^{o}C for 24

hours. The samples were sealed in screw-capped tubes (Kimex 16x100mm with teflon-coated caps) and heated in an oven (Fisher 300). At the end of the time period, the tubes were allowed to cool. They were then opened and heated in a sand bath at 300-350^{o}C until the nitric acid had evaporated (6-7 hours). The residue was dissolved in 1ml de-ionized water and transfered to a plastic tube. The washing and transfers were repeated twice. The rest of the protocol was the same as that for inorganic sulfate determination starting with the addition of the barium chloride and hydrochloric acid.

Four compounds were used for the recovery experiments: indoxyl sulfate, chondroitin sulfate A, heparin and dehydroisoandrosterone 3-sulfate. All compounds are sulfate esters and are found in urine. Indoxyl sulfate, an indole derivative, was selected as a representative phenolic sulfate. Chondroitin sulfate and heparin are both carbohydrate sulfates but heparin also contains a sulfamate linkage. Dehydroisoandrosterone 3-sulfate was included as a recovery standard because certain steroid and aliphatic alcohol sulfoconjugates are resistant to hydrolysis (42, 43). Therefore, the recovery of this steroid sulfate indicated the general applicability of this method to sulfoconjugate quantification.

Mean recoveries for indoxyl sulfate, chondroitin sulfate A, heparin and dehydroisoandrosterone 3-sulfate were 121.7%, 149.2%, 144.4% and 139.3%, respectively (see Appendix: Critique of Methodology 3. Recovery Compound Determination for data). Since recoveries were consistently greater than 100%, each day's analysis included indoxyl sulfate-spiked and nonspiked urine samples. The calculated concentration for a subject's sample was adjusted according to the recovery determined for that day's run.

The inorganic sulfate concentration of acid-hydrolyzed urine minus that of the non-hydrolyzed urine equals the amount of sulfoconjugates in that sample. Results were expressed in terms of bound (conjugated) sulfate and total sulfate excreted per 24 hours and per mole creatinine. The latter expression standardizes the amount of sulfoconjugates excreted to a relatively constant breakdown product of an endogenous compound.

Creatinine Analysis

Creatinine was determined in duplicate using a Technicon AutoAnalyzer. The procedure is based on the reaction between the urine sample, sodium hydroxide and picric acid (Jaffe reaction). A continuous flow of urine was segmented

by an air bubble between each sample. The air bubble maintained the integrity of the samples and improved the mixing of urine with the reagents.

The urine was diluted 1:5 before it entered the AutoAnalyzer system. There, it was diluted further with 1.8% sodium chloride. When it left the dialyzer, 0.5N sodium hydroxide was mixed in. Then saturated picric acid was added and mixed. The colorimeter read the absorbance of the mixture at 505nm in a 15mm flowcell. The sample creatinine concentration was determined from a standard curve plotted from concentrations ranging from 0.2mg/ml to 1.0mg/ml creatinine, and corrected for the dilution factor.

Selection of Samples for Analysis

One day was randomly selected and analyzed for each subject that complied with the collection protocol. For assessing the effect of intra-variability, all three days for a randomly selected subgroup of forty subjects were analyzed as well.

Inadequate subject compliance was defined as data which were greater than two standard deviations from the mean creatinine excretion (mg/24 hr/kg body weight) within and between subjects. The data which passed the criteria were the sample from which random selections were made.

RESULTS

Subjects

One hundred thirty-five subjects complied with the collection protocol which included refraining from taking any medication during the collection period. All subjects passed the intra- and inter-variability tests conducted on creatinine excretion as described in the "Material & Methods" Section. Subject ages ranged from 18 to 47 years with a mean of 20.7 years. One day's urine from each subject in this group was randomly selected and analyzed. Figures 5 and 6 are the side-by-side boxplots of bound sulfate and total sulfate per 24 hours and per mole creatinine. Table 3 lists the ranges and mean excretion for these variables.

Forty subjects were randomly selected from the sample and all three days of urine analyzed. Figures 7 and 8 are the side-by-side boxplots of bound sulfate and total sulfate per 24 hours and per mole creatinine. Table 4 lists the ranges and mean excretion for the above variables. The intra- and inter-variability and corresponding Coefficient of Variation (CV) for the three-day analysis are listed in Table 5.

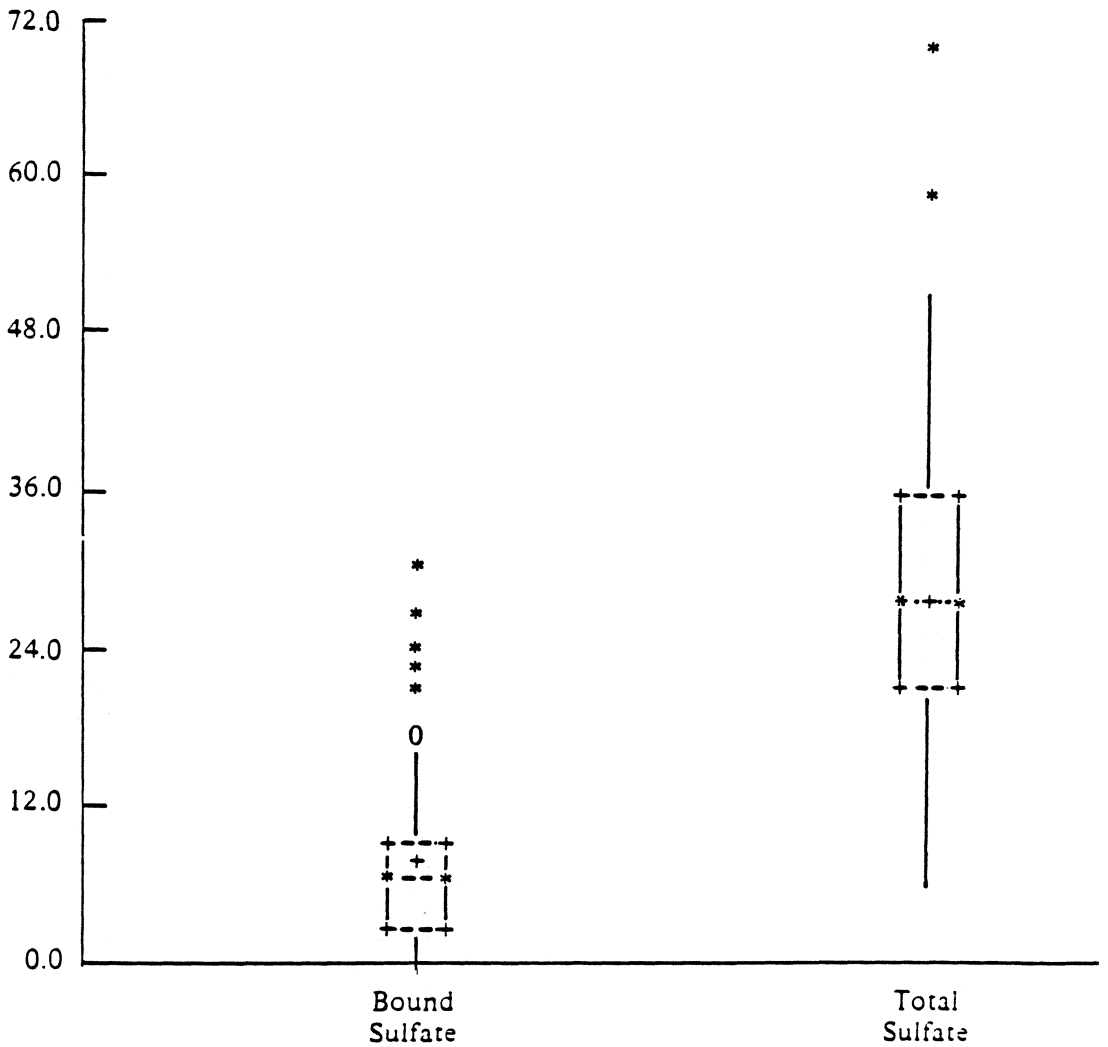


Figure 5. Boxplots (o2) of bound sulfate and total sulfate (mmole sulfate/24 hours) for the one-day sample.

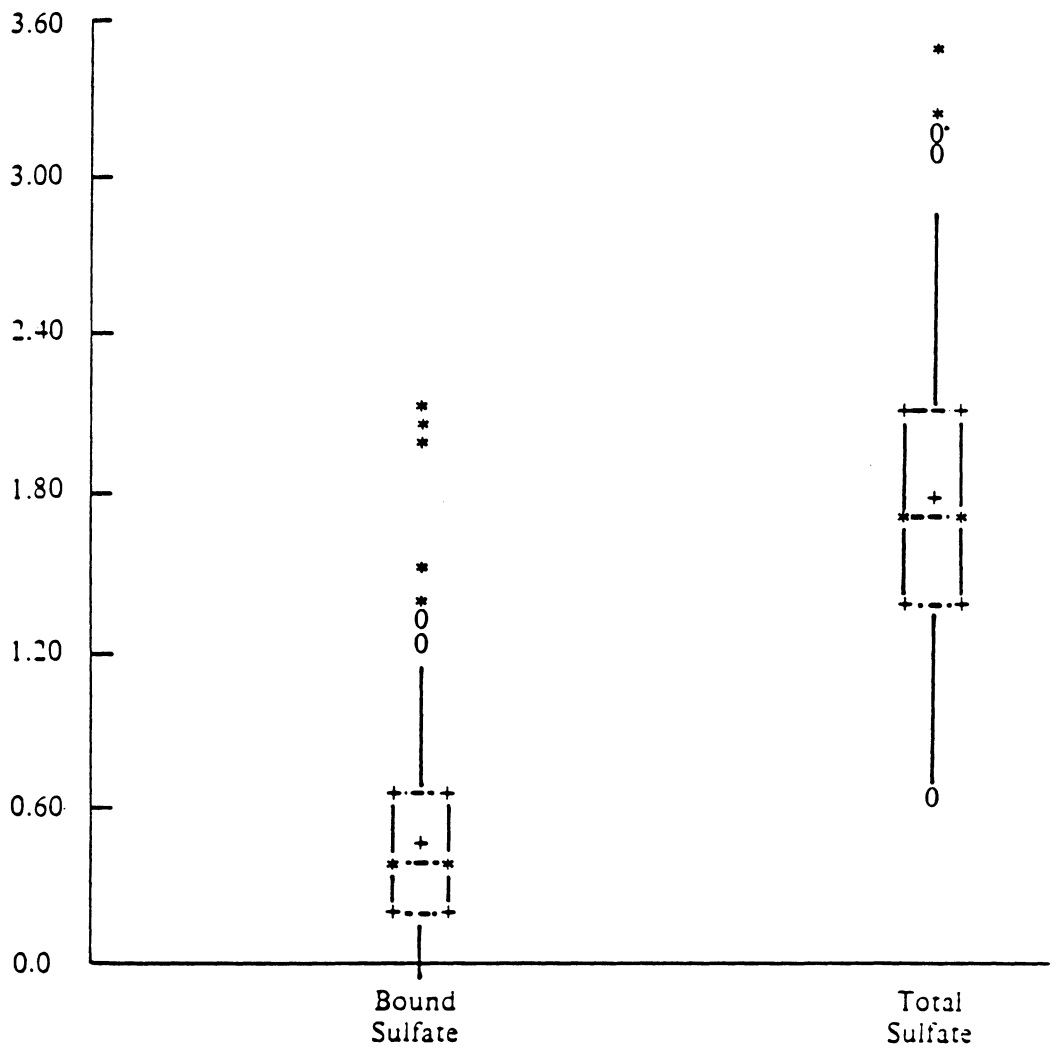


Figure 6. Boxplots (62) of bound sulfate and total sulfate (mole sulfate/mole creatinine) for the one-day sample.

Table 3.

Urinary Excretion of Bound Sulfate and Total Sulfate for the One-Day Sample

Analyte	Excretion	
	Mean(SD)	Range
Bound Sulfate		
mmole/24 hr.	7.45(5.62)	0.21-31.27
mole/mole creatinine	0.49(0.40)	0.01-2.15
Total Sulfate		
mmole/24 hr.	28.53(10.74)	6.77-71.29
mole/mole creatinine	1.81(0.58)	0.66-3.54

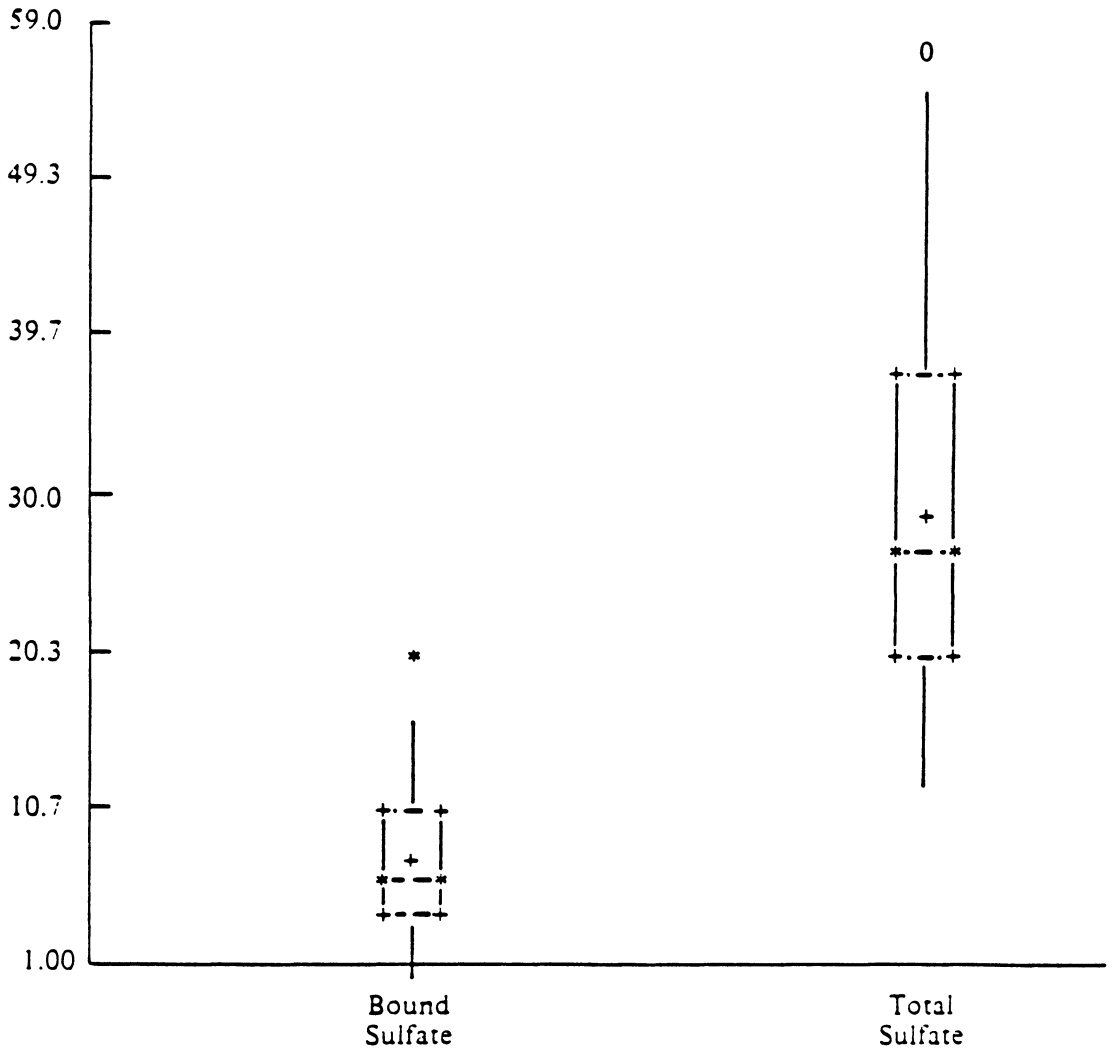


Figure 7. Boxplots (62) of bound sulfate and total sulfate (mmole sulfate/24 hours) for the three-day sample.

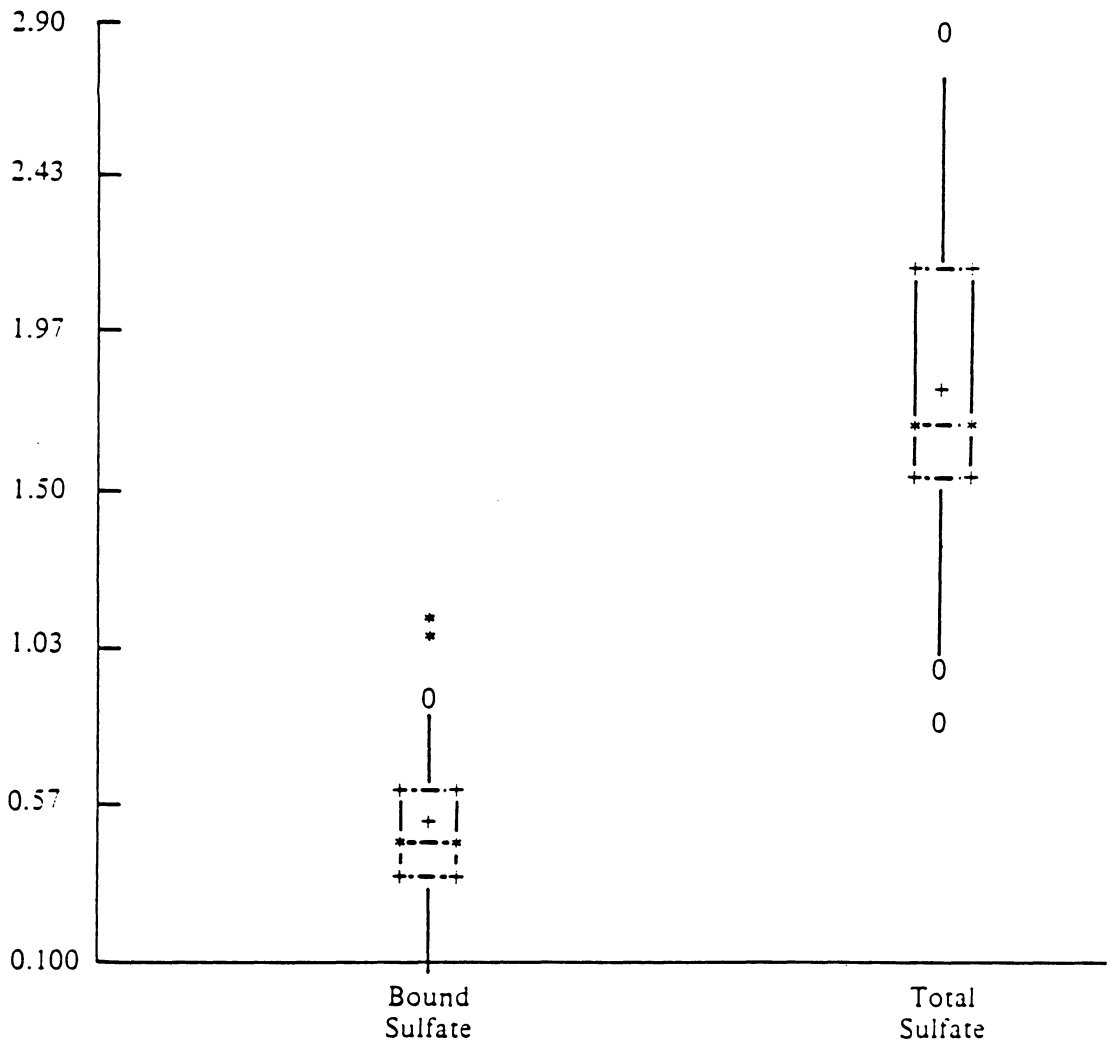


Figure 8. Boxplots (62) of bound sulfate and total sulfate (mole sulfate/mole creatinine) for the three-day sample.

Table 4.

Urinary Excretion of Bound Sulfate and Total Sulfate for the Three-Day Sample

Analyte	Excretion	
	Mean(SD)	Range
Bound Sulfate		
mmole/24 hr.	7.65(5.34)	0.12-26.42
mole/mole creatinine	0.49(0.35)	0.01-2.09
Total Sulfate		
mmole/24 hr.	28.92(12.39)	4.71-71.29
mole/mole creatinine	1.81(0.58)	0.66-3.54

Table 5.

Variation in Urinary Excretion of
Bound Sulfate and Total Sulfate
For the Three-Day Sample

Analyte	Variation[a]			
	Intra	CV[b]	Inter	CV
Bound Sulfate				
mmole/24 hr.	4.39	57.42	6.89	90.03
mole/mole creatinine	0.32	65.67	0.40	81.38
Total Sulfate				
mmole/24 hr.	6.58	22.76	19.48	67.35
mole/mole creatinine	0.43	23.83	0.81	44.90

[a]Intra- and inter-variation reported as standard deviation.
[b]CV = coefficient of variation (%).

Diet

Since only two subjects out of one hundred thirty-five identified themselves as vegetarians, the factor of vegetarianism was not given further consideration. As a matter of note, the two aforementioned subjects were not among the randomly selected three-day sample of forty subjects.

By using food exchange lists (44) for analysis of the food frequency questionnaire, the number of servings for each food group was converted into kilocalories and grams of protein, carbohydrate and fat per day. The subjects consumed an average of 2370.59 kilocalories (assuming 15% due to alcohol intake), 113.43 grams of protein, 291.06 grams of carbohydrates and 50.42 grams of fat per day. When calculated according to the energy content of the diet, 18% was from protein; 49%, from carbohydates; and 18%, from fat.

Regression analysis did not reveal a significant correlation between sulfate excretion and protein intake or with the number of meat and vegetable servings per day.

Other Environmental Effects

The excretion of bound sulfate and total sulfate per 24 hours for various environmental factors for the one-day sample is presented in Table 6. Nonparametric statistical

Table 6.

Urinary Excretion of Bound Sulfate and Total Sulfate According to Certain Environmental Factors For the One-Day Sample

Parameter	n	Excretion (mmole/24 hr.)[a] Bound	Total
Cancer in Family			
Yes	74	7.01(5.68)	28.19(10.19)
No	61	7.97(5.55)	28.95(11.44)
Cigarette Smoker[b]			
Yes	19	6.50(6.04)	26.83(10.91)
No	113	7.60(5.61)	28.81(10.82)
Marijuana Smoker			
Yes	36	6.30(5.24)	26.80(9.94)
No	99	7.86(5.71)	29.16(11.00)
Beer Consumer			
Yes	123	7.63(5.72)	29.13(10.89)[c]
No	12	5.56(4.11)	22.39(6.80)
Took Medication During Previous Month			
Yes	111	7.68(5.78)	29.02(11.14)
No	24	6.37(4.74)	26.27(8.53)
Took Analgesics During Previous Month			
Yes	91	7.45(5.66)	28.96(10.35)
No	44	7.43(5.59)	27.64(11.58)
Exercise			
Light	33	7.07(6.38)	27.08(10.86)
Mild	45	7.24(5.01)	28.59(10.39)
Moderate	48	7.13(5.06)	28.32(9.72)
Vigorous	9	11.53(7.57)	34.69(16.12)
Participate in Marathon Races			
Yes	13	9.13(4.23)[d]	31.69(10.20)
No	122	7.27(5.73)	28.20(10.78)

[a]Excretion values reported as mean(SD).
[b]Some subjects did not answer this question.
[c]Significant difference ($p<0.05$).
[d]Marginal significant difference ($p<0.10$).

procedures were performed because the distributions of excretion were skewed and some of the category sizes were small. The Wilcoxon 2-Sample Test was performed on the means for the categories under each environmental factor except "Exercise" which was analyzed by the Kruskal-Wallis Test. Based on these tests, a significant difference ($p<0.05$) between beer consumers and non-beer consumers for total sulfate excretion was found.

Because both bound sulfate and total sulfate had five subjects which consistently appeared as outliers for the considered environmental factors, the sample was appropriately trimmed to one hundred twenty-five subjects for each variable. The same statistical procedures were performed on the trimmed sample. Once again, only the total sulfate excretion was found to be significantly greater ($p<0.05$) in beer consumers than in non-beer consumers. The marginal significant difference ($p<0.10$) between marathon racers and non-racers disappeared with the trimmed sample.

Because the large intra-individual variation may have "masked" any differences in the one-day data, the tests were performed on the subject means of the three-day sample (see Table 7). For this sample, beer consumers excreted significantly more ($p<0.05$) bound sulfate and total sulfate than non-beer consumers; cigarette smokers excreted

Table 7.

Urinary Excretion of Bound Sulfate and Total Sulfate According to Certain Environmental Factors For the Three-Day Sample

Parameter	n	Excretion (mmole/24 hr.)[a] Bound	Total
Cancer in Family			
Yes	24	6.88(3.58)	26.69(10.78)
No	16	8.80(4.36)	32.28(11.43)
Cigarette Smoker[b]			
Yes	4	5.31(2.12)	20.65(8.78)[c]
No	35	7.95(4.12)	29.89(11.37)
Marijuana Smoker			
Yes	10	5.71(2.38)[c]	23.21(8.77)[c]
No	30	8.30(4.22)	30.83(11.46)
Beer Consumer			
Yes	34	8.14(3.99)[d]	30.34(11.50)[d]
No	6	4.90(2.72)	20.88(4.82)
Took Medication During Previous Month			
Yes	32	8.25(4.12)[c]	30.26(11.90)
No	8	5.24(2.14)	23.60(6.11)
Took Analgesics During Previous Month			
Yes	14	7.02(4.71)	28.31(12.24)
No	26	7.99(3.57)	29.25(10.92)
Exercise			
Light	10	8.24(4.59)	30.31(11.95)
Mild	16	7.24(2.79)	27.15(9.55)
Moderate	10	6.80(3.57)	27.27(11.49)
Vigorous	4	9.95(7.32)	36.70(15.87)
Participate in Marathon Races			
Yes	3	8.66(4.19)	29.10(16.33)
No	37	7.57(4.01)	28.91(11.06)

[a]Excretion values reported as mean(SD).
[b]One subject did not answer this question.
[c]Marginal significant difference ($p<0.10$).
[d]Significant difference ($p<0.05$).

slightly less ($p<0.10$) total sulfate, as did the marijuana smokers who also excreted slightly less ($p<0.10$) bound sulfate; and subjects who had taken medication during the previous month excreted slightly more ($p<0.10$) bound sulfate but not total sulfate.

DISCUSSION

Sulfate Excretion

The first objective of this study was to determine the amount of sulfoconjugates excreted in a free-living male population. The excretion patterns of bound sulfate (sulfoconjugate) and total sulfate for the one-day sample were skewed to the right. Lundquist et al. (38) reported excretion values for 10 healthy free-living men. This study's values for total sulfate agreed with the values reported by Lundquist et al.; however, the bound sulfate values were significantly different (see Table 8).

The difference in bound sulfate may be a result of Lundquist et al.'s small sample size, yet environmental factors may have influenced a higher excretion in this study's population. This supposition is strengthened by the fact that total sulfate was very similar in both studies. Experimental factors such as the dietary supply of sulfur and the analytical methods of sulfate determination may be considered comparable, as indicated by the comparable total sulfate excretion. Of course, there is also the possibility that this study had a more efficient hydrolysis procedure resulting in higher bound

Table 8.

Urinary Excretion of Bound Sulfate and Total Sulfate for Two Studies

Analyte	Excretion[a]	
	Lundquist et al.[b]	This Study
Bound Sulfate		
mmole/24 hr.	1.77(0.39)	7.45(5.62)[c]
mole/mole creatinine	0.11(0.02)	0.49(0.40)[c]
Total Sulfate		
mmole/24 hr.	28.07[d]	28.53(10.74)
mole/mole creatinine	1.73	1.81(0.58)

[a]Excretion values reported as mean(SD).
[b]Excretion values reported for 10 male subjects (38).
[c]Significant difference ($p<0.001$).
[d]Standard Deviation for these values were not reported.

sulfate measurements and that the similar total sulfate measurements were a result of high sulfate ingestion by Lundquist et al.'s subjects.

The excretion patterns of bound sulfate and total sulfate for the three-day sample were also skewed to the right, but not to the same extent as the one-day sample. The mean excretions were not significantly different between the one-day and three-day samples. Unfortunately, no studies were found in the literature to which to compare the mean sulfate excretion of repetitive subject sampling.

Dietary Influence

The information provided by the food frequency questionnaire did not provide a clear indication of normal dietary intake for the population. The very nature of a food frequency questionnaire is to provide qualitative, and not quantitative, information. This dietary methodology is primarily useful for studies where individual nutrient data, particularly for moderate to large sample sizes, is not needed (45). There are indications, though, that food frequency questionnaires assist in accurately estimating food intake (46). The accuracy of this study's questionnaire data was most likely compromised by the lack of subject training in serving size determination.

However, since the subjects appeared to be healthy, it can be assumed that they were consuming an adequate diet.

It is interesting to note that correlation analysis did not reveal a significant relationship between protein intake and sulfate excretion. Since protein is composed of sulfur-containing amino acids, and urinary ester and inorganic sulfate excretion decreases upon decreased consumption of these amino acids (47, 48), a positive correlation might naturally be expected. The lack of correlation may be a result of non-quantitative dietary data.

The numbers of meat and vegetable servings were considered since meat is rich in sulfur-containing amino acids and vegetables are a source of sulfation substrates, i.e. phenolic compounds. Once again, significant relationships with sulfate excretion were not observed.

Other Environmental Factors

Other than diet, environmental factors considered were familial cancer incidence, alcohol intake, tobacco smoking, marijuana smoking, medication and specifically analgesic use, and exercise. Of these environmental factors, beer consumption was the only factor to reveal an effect on total sulfate excretion. However, no difference in bound sulfate excretion was observed.

Cigarettes and alcohol were chosen as environmental factors because according to Johnston et al. (49), they are used by eighteen year old students more than illicit drugs. Of the illicit drugs, marijuana is the most prevalent. Of this study's sample of one hundred thirty-five subjects, only 14.4% and 26.6% had tried or used cigarettes and marijuana, respectively. These low figures may be a result of the downward trend of use observed by Johnston et al. (50). They also reported a high consistent rate of monthly alcohol consumption (70.6%) among students during their seven-year test period. This study was consistent with that observation since 91.1% of the subjects drank beer in the previous month.

Alcohol consumption was not expected to influence the excretion of bound sulfate because studies of ethanol administration to rats and isolated heptaocytes did not measure a change in the sulfation of the drug harmol (7). It is interesting to note that a difference in total sulfate excretion was observed. However, this observation may be a result of the fact that the number of non-beer drinkers was considerably smaller than the number of beer drinkers.

As for cigarette smoking, a higher excretion of bound sulfate could have occurred since benzo[a]pyrene, a component of cigarette smoke, is detoxified by sulfation

(51). This study had no expectations on marijuana smoking and sulfation since no data is present in the literature. In either case, no differences were observed between smokers and non-smokers.

A question on the familial cancer incidence was included on the questionnaire as a general estimation on whether or not the subjects could be characterized as being unusually susceptible to some form of cancer. This susceptibility could be considered as a genetic predisposition for the disease. The sample was almost equal in this category since just a little more than half of the subjects (54.8%) had at least one parent or grandparent with cancer. The bound sulfate and total sulfate excretions were not significantly different between subjects with and without cancer in their families.

Medication was a natural environmental factor to consider since these compounds definitely undergo xenobiotic metabolism. In addition several drugs are known to induce Phase I reactions. Analgesics were specifically looked at because several of these compounds are detoxified by sulfation. Consequently, most sulfation research has been conducted on this class of compounds. Once again no effect on the sulfate excretions was observed.

Exercise was considered because the activity level of an individual influences his health. In addition the

possibility that vigorous workouts could result in significantly higher sulfate excretion due to increased protein catabolism from muscle breakdown was considered.

Approximately the same number of subjects described their exercise regime as mild or moderate, 33.3% and 35.6%, respectively. A light activity level was claimed by 24.4%, and only 6.7% had vigorous workouts. Even though the subjects with vigorous exercise regimes excreted more bound sulfate and total sulfate than the other subjects, the differences were not significant. However, a marginally significant difference ($p<0.10$) was observed in bound sulfate excretion in marathon participants. Once again, the considerably smaller number of marathon participants (9.6%) may be distorting the true relationship between sulfate excretion and marathon activity.

Variability

All of the previous discussion was based on the one-day sample. To determine the effect of intra- (within subject) and inter- (between subject) variability, three days for forty subjects were analyzed. The coefficient of variation, which measures the standard deviation proportional to the mean, was in all cases quite large. This high variability indicates that the means are not very

precise and that the true relationships for all of the environmental factors may be masked.

A large inter-individual variation was expected because of the considerable number of genetic and external influences to which a person may be subjected (see Figure 9). It has been estimated that drug elimination rates may vary as much as 40-fold depending on the drug and population studied (52).

Caldwell et al. (53) measured the inter-individual variation in the sulfation and glucuronidation of paracetamol (acetaminophen) and salicylamide. After oral administration of a drug, eight-hour urine samples were collected from thirty-eight male and twenty-four female human subjects. The paracetamol frequency distribution of the % total recovery as the glucuronide/% total recovery as the sulfate (G/S ratio) was skewed to the right. The G/S ratio varied 13-fold (ranged from 0.35 to 4.64) among the subjects. In addition four of the thirty-four subjects who took this drug were relatively unable to form the sulfate conjugate. Reproducibility was confirmed by re-testing ten subjects.

The salicylamide frequency distribution of the G/S ratio was normal, and the ratio varied 16-fold (ranged from 0.26 to 4.26) among the subjects. Once again, a few of the subjects excreted very low levels of sulfate conjugates.

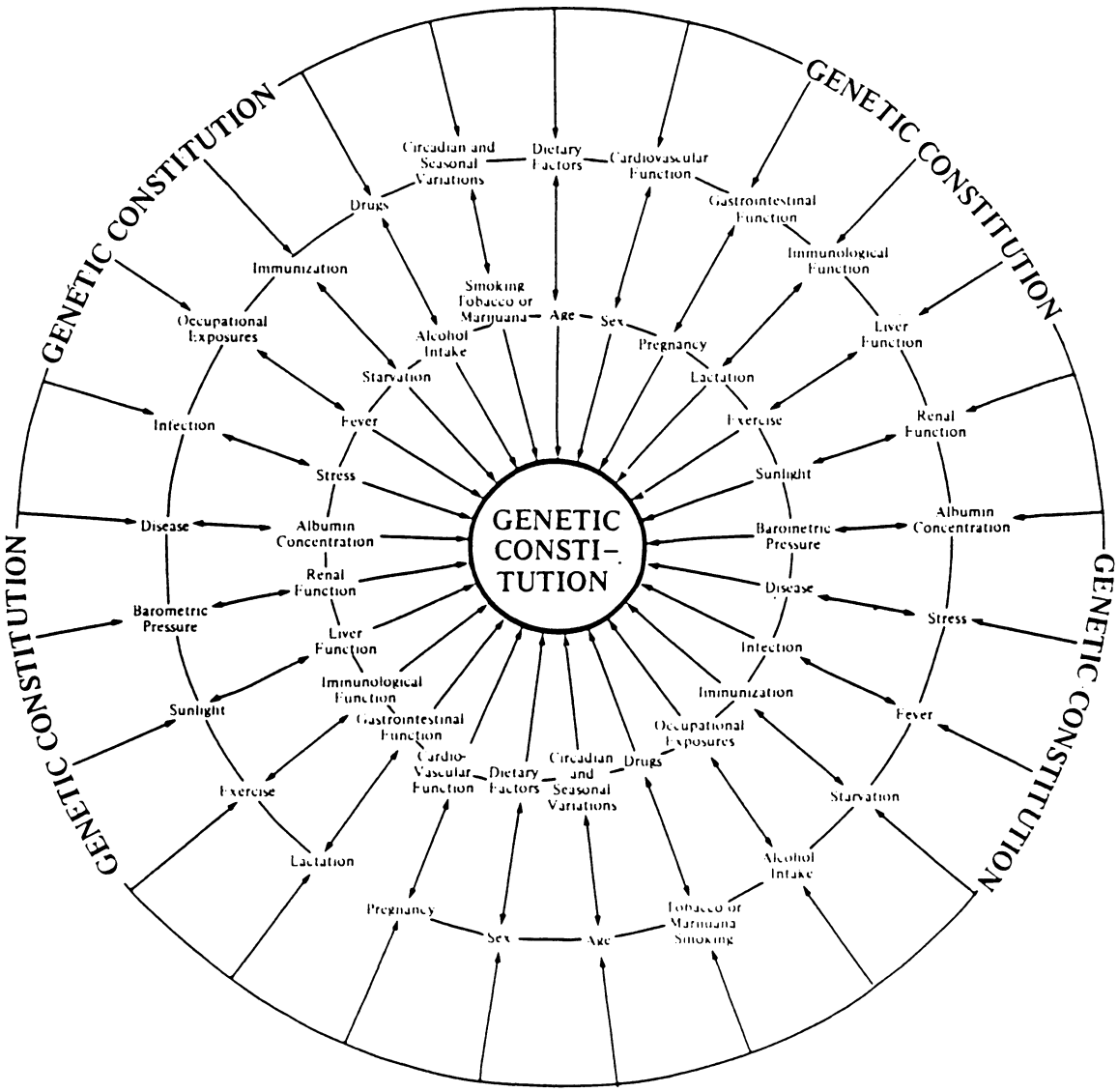


Figure 9. Interaction between environmental factors and genetic constitution. This design is speculative and is not intended to depict the current state of knowledge in the field.

[Adapted from (52)]

Six subjects, selected to represent the middle and extremes of the distribution, were re-tested and were found to have reproducible metabolic profiles.

A comparison between % dose eliminated and % eliminated as glucuronide, sulfate or G/S ratio revealed that the increased sulfoconjugate formation resulted in increased paracetamol elimination. In addition the G/S ratio was negatively correlated with this drug's elimination which supports the previous finding. Salicylamide elimination does not seem to be dependent on the nature of the conjugates formed since no correlations were significant.

Caldwell et al. (53) concluded that even though the drugs in question are structurally similar, their metabolism occurs under different controls. Apparently, the relative inability of a few subjects to produce sulfate conjugates is an important factor in the large inter-individual variation calculated for both drugs.

This study's determinination of inter-individual variation for bound sulfate and total sulfate is unique in the literature. It should be noted that the variation for total sulfate excretion was less than that for bound sulfate excretion. Because of the design of this study, it is impossible to determine a predominant factor which is responsible for the large inter-individual variation.

Alvares et al. (54) investigated intra-individual variation by administering one of three drugs - phenylbutazone, antipyrine and phenacetin - to four male and three female human subjects five times at six-week intervals. There was a two-week interval between test drug administration.

Plasma half-lives were determined for the three drugs. In addition the area under the curve (AUC) which is related to clearance was calculated for phenacetin. Table 9 contains a summary of the intra- and inter-individual coefficients of variation for these drugs.

As expected the inter-individual variation was quite large depending on the variable considered, as in the case of phenacetin AUC. The intra-individual variation was also large. For phenacetin, both half-life and AUC measurements, the intra-individual variation was larger than the inter-individual variation. Alvares et al. interpreted the finding as an indication that the environment influences phenacetin metabolism more than phenylbutazone and antipyrine metabolism. According to Vesell and Penno (55), the magnitude of intra-individual variation is "low for drugs with low hepatic extraction ratios (such as phenylbutazone and antipyrine) and high for drugs with high hepatic extraction ratios (e.g. phenacetin)."

Table 9.

Coefficients of Variation for Three Drugs

Parameter	Intra-Individual Coefficient of Variation (%)	Inter-Individual Coefficient of Variation (%)
Phenylbutazone half-life	8.66	17.29
Antipyrine half-life	13.49	24.29
Phenacetin half-life	19.50	4.40
AUC	58.81	54.93

AUC = area under the curve.

Adapted from (54).

Phenacetin AUC is an index of first-pass metabolism which, in this case, is more variable than plasma half-life. Factors which stimulate first-pass metabolism of phenacetin are cigarette smoking, charcoal-broiled meat ingestion and cruciferous vegetable ingestion (54).

As a precaution, the assay's variability was tested and determined to be no more than 5.7%. Thus the assay could not account for all or even most of the observed variation.

Caldwell et al. (53) reported intra-individual variations of 18% and 8% for paracetamol and salicylamide elimination rates, respectively. This study determined variations of 57% for bound sulfate and 66% for total sulfate excretion in 24 hours. Vesell and Penno (56) stated that intra-individual variation is often erroneously considered to be negligible as compared with inter-individual variation. As this study's findings and those of the cited articles indicate, intra-individual variation is a significant factor to consider.

The magnitude of inter-individual variation depends on genetics, environmental perturbations, type of drug tested and the measured parameter (52, 56, 57). Factors which affect the magnitude of intra-individual variation are, with the exception of genetics, all of the above (58). The design of any experiment is very important since both types of variation are affected by factors which may not be

normally considered, including circadian (59) and seasonal changes (60).

An example of circadian effects on sulfation would be the significant reduction in sulfotransferase activity of rat liver at 2100 hour as opposed to 0900 hour (61). In the same study, fasting eliminated the diurnal variation in sulfotransferase activity but induced the same type of variation in sulfatase activity. This last fact illustrates the importance of diet and temporal considerations in experimental designs.

In order to minimize the effect of intra-individual variation, the subject means of the three-day sample for this study were tested for environmental significance. The few differences observed could be attributed to the small sample size. Once again there was not a clear indication of a factor's effect on sulfation.

Further research is needed to clarify the sulfation excretion pattern of a free-living population. Because of the large magnitude of variation present in this study, another study with fewer subjects but more collection days is recommended. In addition if the objective is to identify factors which affect sulfation, more control on the subjects' diet and lifestyle is indicated.

SUMMARY AND CONCLUSIONS

The amount of sulfoconjugate excreted by a free-living male population and the effect of certain factors, i.e. familial cancer incidence, alcohol intake, tobacco smoking, marijuana smoking, medication and specifically analgesic use, exercise, protein intake and the number of meat and vegetable servings consumed daily, were investigated. One hundred thirty-five subjects complied with the collection protocol. One day of urine was analyzed for each subject and three days of urine were analyzed for a randomly selected sub-group of forty subjects.

All excretion patterns were skewed to the right. For the one-day sample, the average amount of bound sulfate excreted was 7.45 mmole/24 hr. or 0.49 mole/mole creatinine. Total sulfate was 28.53 mmole/24 hr. or 1.81 mole/mole creatinine. The values for the three-day sample were comparable.

An investigation on the one-day data on the relationship between sulfate excretion and protein intake or with the number of meat and vegetable servings per day did not reveal a significant correlation. Of the other environmental factors under consideration, only beer

consumption appeared to elicit a change in sulfate excretion. For total sulfate exclusively, a higher excretion was observed among beer drinkers, which may be due to the small number of subjects who did not drink beer. Trimming the sample to minimize the influence of a few outliers did not alter the results of the statistical tests.

Analysis of the three-day data revealed a large degree of variability. Bound sulfate had intra- (within subject) and inter-individual (between subject) coefficients of variation of 57.42% and 90.03% for mmole/24 hr. For total sulfate intra- and inter-individual coefficients of variation were 22.76% and 67.35%, respectively.

In an attempt to minimize the effect of intra-individual variation and to ascertain possible effects of environmental factors on sulfate excretion, the three-day data were subjected to the same statistical tests as the one-day group. Once again, the only significant difference was observed for beer consumption.

Since studies on variation are few in number, it is possible that sulfation's variation may be as large as this study indicates. On the other hand, the phenonmenon may be peculiar to the tested sample. The necessity for further research regarding sulfoconjugate excretion and genetic and environmental influences is indicated.

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APPENDICES

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

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

I. SUBJECT REQUIREMENTS:

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

II. PROCEDURES:

A. FOOD FREQUENCY QUESTIONNAIRE: You will be required to complete a food frequency questionnaire, identical to the one submitted at the Pre-Experimental screening session.

B. URINARY SAMPLES: You will be required to collect all urinary excretion produced during a consecutive three day period. The collection will consist of three, 24-hour samples, to be submitted to the lab at the end of each 24-hour period. You will be provided with the necessary urine bottles and complete instructions for collection and proper handling.

III. POSSIBLE RISKS:

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

IV. POSSIBLE BENEFITS:

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

V. COMPENSATION:

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

VI. YOUR RIGHTS:

A. You have the right to confidentiality. All information obtained during this study that can be identified with you, shall remain confidential.

B. You have the right to expect an honest answer to any questions that you may have at anytime during the study.

C. You are free to withdraw from the study at any time, without prejudice.

D. You have the right to receive a copy of this document.

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

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

DATE _____ SIGNATURE OF PARTICIPANT _____

SIGNATURE OF WITNESS _____ SIGNATURE OF INVESTIGATOR(S) _____

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

1. Dr. Ryland E. Webb, Principal Investigator
   Department of Human Nutrition and Foods
   Phone Number 961-6784/5549

2. Dr. Forrest W. Thye
   Department of Human Nutrition and Foods
   Phone Number 961-6620/5549

3. Graduate Students: Jane Santi and Vernice Robichaud
   Phone Number 961-5840

DATE _____
NUMBER _____

DETOXIFICATION PROFILE STUDY
1984
PRE-EXPERIMENTAL SURVEY

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

A. GENETICS

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

   a. Yes b. No

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

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

B. DIETARY

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

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

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

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

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

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

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

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

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

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

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

a. Yes b. No c. Uncertain

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

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

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

C. TOBACCO PRODUCTS

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

a. Yes b. No

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

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

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

Cigarettes	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)						

E. MEDICATIONS

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

   a. Yes b. No

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

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

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

   a. Yes b. No

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

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

F. ENVIRONMENTAL

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

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

EXAMPLE: ACETONE

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

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

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

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

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

   a. Yes b. No.

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

   a. Yes b. No

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

   a. less than 6 months ago
   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

Critique of Methodology

1. Overview

Advantages

"Realistic" study - based on a free-living population

Fast and easy collection of samples

May be used with many subjects

Investigated sulfation in general

Minimal risk to subjects

Disadvantages

No controls

Reproducibility in a population is not established

Extrapolations to the population may be inaccurate since do not know if the subjects really reflect the population from which they are selected

Identification of causal relationships is difficult

Difficult to quantify a broad class of compounds

Sulfoconjugates determined by an indirect measurement

Actually measured barium and not sulfate

Analytical procedure is time consuming with a high repeat rate (25%)

Critique of Methodology

2. Sources of Experimental Error

Indirect measurement for sulfoconjugate determination

Measured barium and not sulfate

Faulty freeze drier - samples thawed and were pulled up the length of the test tube by the vacuum

Potential loss of sample during water transfer from a glass tube to a plastic tube

Potential loss of precipitate while discarding the supernatant

Used an etched line on the tubes as a volume indicator

Potential loss of sample while pipetting - 0.2ml and 1ml aliquots

Critique of Methodology

3. Recovery Compound Determination

	Indoxyl Sulfate	Chondroitin Sulfate	Heparin	Dehydroisoandrosterone Sulfate
	125.2%	146.4%	144.6%	147.9%
	132.7%	171.4%	153.9%	149.3%
	125.2%	146.4%	144.6%	147.9%
	103.5%	132.4%	134.5%	112.1%
Mean	121.7%	149.2%	144.4%	139.3%

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