The effect of a semisynthetic diet on the profile of urinary conjugates in male adults

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The effect of a semisynthetic diet on urinary conjugate levels was determined in 18 male adults (22 to 40 years). Subjects consumed a self-selected diet for 3 days and a semisynthetic diet for 7 days. Glucuronides and mercapturates were quantified spectrophotometrically using naphthoresorcinol and Ellman's reagents, respectively. Atomic absorption was used to measure barium chloride-precipitated sulfate for sulfoconjugates. Conjugated amino acids were determined by high performance liquid chromatography of phenylisothiocyanate derivatives. A multivariate analysis of variance was used to compare the means of the dietary periods, and a repeated measures analysis with Helmert transformation was used to determine response to dietary change. Mercapturates and amino acid conjugates were most sensitive to dietary change, with quantities excreted decreasing by about 50% during the semisynthetic diet period (0.29 versus 0.16 mmol/24 hr; 5.99 versus 3.06 mmol/24 hr, respectively). Glucuronides were the least responsive to dietary change, with no significant difference between the means of the two diet periods (self-selected diet, 2.98; semisynthetic, 3.05 mmol/24 hr). Sulfoconjugate levels exceeded those of the other conjugates measured. Sulfoconjugates were initially decreased on the semisynthetic diet (5.28 versus 3.98 mmol/24 hr), but by day 4, sulfo-conjugate excretion began to increase. In summary, the quantity of conjugates excreted were found to be sensitive to dietary changes, with some pathways more responsive than others. (J. Nutr. Biochem. 5:451–456, 1994.)

Keywords: urinary conjugates; semisynthetic diet; glucuronides; sulfoconjugates; mercapturates; amino acid conjugates

Introduction

One of the factors that most influences metabolism and the disposition of exogenous chemicals is nutrition.[1–5] All the reactions of xenobiotic metabolism are dependent on the nutrient supply of the body.[6,7] Mammals are constantly exposed to naturally occurring and synthetic chemicals that are toxic or carcinogenic via ingestion of foods.[5] Because diet for the human is so varied, it is the most complex variable in ascertaining its role in the rates of metabolic activation or detoxication of drugs and xenobiotics.

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

Because of the relationship between toxicology and nutrition, Truhaut and Ferrando[11] recommended that when dealing with toxicologic experiments, it is advisable to account for nutritional causes capable of altering the body reactions to toxic substances. Thus, Abbott et al.[12] compared the effect of purified and commercial crude diets on the components of the hepatic mono-oxygenase system of rats. Little or no consistent diet-related difference was observed, nor were results significantly less variable with either diet. These results differ from studies dealing with humans, and the effect of diet on xenobiotic metabolism. Several studies have shown that the magnitude of intraindividual variation is not always small.[13,14] The more subjects are environmentally perturbed, the larger the magnitude of intraindividual relative to interindividual variability.[15] Murano et al.[16] found that it was difficult to make any definite conclusions because of

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the large intra-individual variability within his subjects. The discrepancy between human and animal studies may be due to the fact that the human diet is so much more varied than that of laboratory animals. Furthermore, human food may contain mutagens because it is usually cooked, and these products are unlikely to be in the diets of laboratory animals.[4] Therefore, a greater difference in effect on xenobiotic metabolism would be expected in humans when a varied, self-selected diet is consumed as compared with consumption of a semisynthetic diet.

Most research dealing with the effect of diet on drug (xenobiotic) metabolism has focused on phase I reactions. Few researchers have looked at the effect of diet on the conjugation (phase II) reactions. The purpose of this study was to determine the influence of food on conjugate excretion by measuring conjugate levels of individuals consuming a semisynthetic diet that minimizes exposure to dietary xenobiotics and to determine the time required for conjugate levels to respond to the diet modification. Thus, a semisynthetic diet would be expected to reduce exposure to xenobiotics because diet represents the major source of exposure to foreign chemicals in humans and probably accounts for a considerable portion of intraindividual variability. The semisynthetic diet also would standardize dietary intake for each subject from day to day, thereby further reducing variability.

Methods and materials

Subjects

The subjects were 18 male Caucasian students at Virginia Polytechnic Institute and State University. Subjects were between the ages of 22 and 40 years and selected from a number of respondents to a questionnaire concerning health; smoking and drinking habits; and willingness to abstain from alcohol, tobacco, and drug use. Subjects continued normal daily activities during participation in the study, except that no drug or medication was allowed and that, from the fourth day until completion of the study, a semisynthetic liquid diet was consumed as a total replacement for normal dietary foods. Feeding was on a demand basis without restriction as to time.

Diets

During the first 3 days, subjects consumed their usual, self-selected diets and kept records of food consumption for the purpose of determining average caloric intake. For the next 7 days, subjects were given a nutritionally adequate, chemically defined liquid diet *(Table 1)*. The semisynthetic diet was selected for its acceptability to humans over a period of several days, capacity to be used as a total feeding regimen, and theoretical lack of possible xenobiotics except for vanilla, a flavoring agent. Subjects were given as many 237 mL cans as were necessary to maintain the caloric intake as calculated from the 3-day self-selected diet. Body weights were measured daily to ascertain that sufficient semisynthetic diet was being consumed by each subject. During the 7 days when subjects were consuming the semisynthetic diet, no other foods or drugs were allowed. No other restrictions were put on the subjects' lifestyles or consumption of water.

Sample collection and analysis

Urine was collected for a total of 10 days in 1-L plastic bottles that had been thoroughly cleaned and autoclaved. Total daily urine volumes were measured and recorded. After mixing the 24 hr urine collection, aliquots were immediately frozen and kept at $-20°$ C for analysis of specific conjugates (glucuronides, sulfoconjugates, mercapturates, and amino acid conjugates).

The modified naphthoresorcinol (NR) method of Mazzuchin et al.[17] was used for quantification of urinary conjugated glucuronic acid. Interference by glucose was selectively eliminated with glucose oxidase, and free glucuronic acid was decomposed to an NR-insensitive product using sodium hydroxide. The methodology permits the determination of conjugated glucuronic acid even though the urine may contain high glucose concentrations and free glucuronic acid. The color developed was extracted with ethyl acetate and the absorbance was measured against a blank at 564 mm (Milton Roy Spectronic 601, Rochester, NY, USA). Concentrations of conjugated glucuronic acid were determined from a standard curve that had been developed using phenolphthalein glucuronide.

Quantification of sulfoconjugates was determined by using a modified method of Lloyd et al.[18] and was calculated as the difference between inorganic sulfate before and after hydrolysis with fuming nitric acid. Inorganic sulfate was precipitated as a barium sulfate pellet, dissolved in EDTA, and measured by atomic absorption (Perkin-Elmer 2100, Norwalk, CT, USA). Varying concentrations of aqueous sodium sulfate, which had been similarly treated, were used to determine the standard curve.

A modified colormetric method of Seutter-Berlage et al.[19] was used to determine mercapturic acids. The characteristic yellow color that developed as a result of using Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) was measured at 412 nm (Milton Roy Spectronic 601). Mercapturic acid concentration was determined by the difference between free sulfhydryls (after reduction with $NaBH_4$) and sulfhydryls after hydrolysis with 5N NaOH. Standard curves for prehydrolysis and posthydrolysis were developed separately using N-acetylcysteine as the standard.

Urinary conjugated glycine, glutamine, and taurine were measured using the method of Bidlingmeyer et al.[20] This method is based on the derivatization of amino acids with phenylisothiocyanate (Edman's reagent) to phenylthiocarbamyl derivatives, which can then be separated by high performance liquid chromatography. The PICO.TAG chromatography system (Water Assoc., Millipore Corp., Milford, MA, USA) was used with a fixed wavelength detector (254 nm) and a solvent system consisting of eluent A (sodium acetate buffer) and eluent B (acetonitrile, methanol, water). The quantification of the conjugated compounds was determined by calculating the differences of amino acid before and after HCl hydrolysis. Due to the high cost of analysis, amino acid conjugate determinations were done on pooled samples consisting of three randomly formed groups, each containing six subjects. Only pooled urines from the third day of the self-selected diet and the second, fifth, and seventh day of the semisynthetic diet were analyzed.

Statistical analysis

Repeated measures analysis of variance, with Helmert transformation,[21] was used to determine the response of the excretion levels of the urinary conjugates to the semisynthetic diet over time. To compare the means of the self-selected diet and the semisynthetic diet, a multivariate analysis of variance was used.[21]

Results

Compliance with the use of the semisynthetic diet and other restrictions was excellent for all subjects. Body weight changes during the semisynthetic diet period were limited to a mean change of -0.65 kg (range, 0.70 to -2.0).

The quantities of urinary conjugates excreted for both the self-selected and semisynthetic diets are shown in *Table 2*. For the self-selected dietary period, the total of amino

Table 1 Composition of semisynthetic diet*

Ingredient	Per 1,000 kcal	Ingredient	Per 1,000 kcal
Protein, g	36.2	Vitamin B_{12}, μg	6.1
Casein (82%)		Nicin, mg	19.6
Soy (18%)		Choline, mg	392
Carbohydrate, g	143.3	Biotin, μg	296
Hydrolyzed cornstarch (61%)		Pantothenic acid, mg	9.81
Sucrose (29%)		Sodium, mg	769
Soy polysaccharide (10%)†		Potassium, mg	1538
Corn oil, g	33.8‡	Chloride, mg	1308
Vitamin A, IU	3269	Calcium, mg	654
Vitamin D, IU	262	Phosphorus, mg	654
Vitamin E, IU	29.6	Magnesium, mg	262
Vitamin K, μg	46	Iodine, μg	98.1
Vitamin C, mg	196	Manganese, mg	3.27
Folic Acid, μg	392	Copper, mg	1.31
Thiamin, mg	1.5	Zinc, mg	14.73
Riboflavin, mg	1.69	Iron, mg	11.77
Vitamin B_6, mg	1.96		

*Enrich with Fiber, Ross Laboratories, Columbus, OH USA.
†Includes 13.1 g total dietary fiber.
‡Includes 15 mg of cholesterol per liter.

Table 2 Urinary conjugates of 18 male adults consuming 3 days of a self-selected diet and 7 days of a semisynthetic diet*

Diet / Day	Conjugates (mmol/24 hr)†			
	Glucuronides	Sulfoconjugates	Mercapturates	Amino acid conjugates‡
Self-selected				
1	2.93 ± 0.94	5.48 ± 3.86	0.26 ± 0.07¶	—
2	2.87 ± 1.03	5.10 ± 2.92	0.28 ± 0.15¶	—
3	2.98 ± 0.96	5.28 ± 4.09	0.29 ± 0.19¶	5.99 ± 0.45‖
Semisynthetic				
1	3.38 ± 1.27	4.52 ± 1.97§	0.15 ± 0.07	—
2	3.18 ± 0.94	4.18 ± 1.92§	0.16 ± 0.08	6.24 ± 0.87
3	3.21 ± 0.80	3.98 ± 1.73§	0.13 ± 0.05	—
4	3.73 ± 1.20	5.20 ± 1.57§	0.13 ± 0.06	—
5	2.85 ± 0.67	5.53 ± 2.11§	0.12 ± 0.04	5.30 ± 0.69
6	3.04 ± 0.72	6.16 ± 2.94§	0.15 ± 0.06	—
7	3.05 ± 0.94	8.52 ± 3.59	0.16 ± 0.05	3.06 ± 0.18

*Enrich with Fiber, Ross Laboratories, Columbus, OH USA.
†Mean ± SD.
‡Only 4 days determined on pooled samples (three groups of six subjects each). Represents the total of glycine, glutamine, and taurine conjugates.
§Each day is significantly different ($P < 0.004$) from the mean of the succeeding days as determined by Helmert transformation of repeated measures analysis.
¶Significantly different ($P < 0.004$) from the mean of the subsequent 7 days of semisynthetic diet.
‖Significantly different ($P < 0.01$) from semisynthetic diet day 7.

acid conjugates, i.e., the total of glycine (4.75 mmol/24 hr), glutamine (1.18 mmol/24 hr), and taurine (0.07 mmol/24 hr) conjugates, was excreted at a mean level of 5.99 mmol/24 hr by the third day. Sulfoconjugate excretion ranged from a mean of 5.10 to 5.48 mmol/24 hr, and the glucuronides ranged from 2.87 to 2.98 mmol/24 hr. Mercapturates were excreted at the lowest level (0.26 to 0.29 mmol/24 hr).

During the semisynthetic diet period, sulfoconjugate excretion reached the highest level of all four pathways measured (8.52 mmol/24 hr). Although amino acid conjugates were not analyzed for the first day of the semisynthetic diet, the quantities of excretion measured for day 2 showed a mean level of 6.24 mmol/24 hr. A decrease occurred by the fifth day (5.30 mmol/24 hr) and, by the seventh day excretion levels dropped precipitously to 3.06 mmol/24 hr. As in the self-selected dietary period, mercapturate excretion (0.12 to 0.16 mmol/24 hr) was the lowest of all the urinary conjugates.

The mean of 3 days of a self-selected diet and the mean of 7 days of a semisynthetic diet for three of the conjugation

pathways measured are presented in *Table 3.* The 7-day mean excretion level (0.14 mmol/24 hr) of mercapturates during the semisynthetic diet was significantly lower ($P < 0.0001$, paired Student's t test) than the 3-day mean (0.27 mmol/24 hr) of the self-selected diet. The mean excretion of conjugates during the self-selected diet for the glucuronides and sulfoconjugates were not found to be significantly different from the mean excretion of these conjugates during the semisynthetic diet.

The excretion levels of the three amino acids measured are represented in *Figure 1.* Glycine is the most predominant amino acid, contributing to more than 70% of the total amino acid conjugate excretion, while taurine excretion contributed less than 5%. Taurine excretion initially increased (more than doubled) while subjects were consuming the semisynthetic diet and later decreased to amounts comparable to the self-selected diet period. Both glycine and glutamine excretion were reduced during the semisynthetic dietary period, with a greater reduction in the glycine excretion levels. Total amino acid conjugate excretion *(Table 3)* was reduced by the seventh day of the semisynthetic dietary period.

Discussion

Mean excretion levels for all four types of conjugates measured while on the self-selected diet were similar to levels previously measured in our laboratory and by other researchers, except for the glucuronides.[16,17,22,23] The values reported by Murano et al.[16] were only 29% of the recorded mean values of the present study for a similar 3-day period. A mathematical correction (using the molecular weight of glucuronate instead of phenylglucuronide) of the former reported values resulted in excretion amounts that conform more with those of the present study (2.93 mmol/24 hr) and with values reported by other researchers, i.e., Muzzuchin et al.[17] (maximum 2.52 mmol/24 hr) and Fishman and Green[23] (maximum 2.38 mmol/24 hr).

Although most of the conjugate types studied responded to dietary changes *(Table 2),* mercapturates seemed the most responsive to the introduction of the semisynthetic diet. Mercapturates were decreased by about 50%. This reduction was immediate and persisted for the duration of the dietary period. The 3 days of the self-selected diet were found to be significantly different ($P < 0.004$) from the mean of the subsequent 7 days of the semisynthetic diet, although there was no significant difference within the 7 days of the semisynthetic dietary period using Helmert transformation for repeated measures analysis. This significant difference was also found using the Student's t test ($P < 0.0001$) *(Table 3).* The lack of significance within the 7 days of the semisynthetic dietary period implied that once excretion levels were decreased they stabilized and remained fairly constant.

Because mercapturates were the most responsive to dietary change, formation of mercapturates would appear to involve mostly exogenous compounds rather than endogenous compounds. Thus, mercapturates appeared to be a better indicator of dietary change than the other conjugates studied. The diet can be a potential source of electrophilic substances, which may have been produced during processing or as a natural product of the food. Once a major source (the self-selected diet) of foreign compounds was removed, the mercapturate excretion decreased and remained low. Van Doorn et al.[24] found that when exposure of one or more electrophilic substances had occurred, urinary mercapturate excretion increased. The reverse, also, would seem to be true—when exposure to electrophilic substances is reduced, urinary mercapturates decrease.

Amino acid conjugate excretion also was responsive to dietary change. Although amino acid conjugates did not respond as quickly as the mercapturates to the dietary change, a decrease was not seen until day 5 of the semisynthetic diet. Excretion levels were reduced by about 50% by the end of the semisynthetic dietary period. Amino acid conjugate excretion on day 7 of the semisynthetic diet was found to be significantly different ($P < 0.01$) from the excretion levels on the third day of the self-selected diet, using the Student's t test *(Table 2).* The drop in the total amino acid conjugate excretion was best represented by glycine excretion levels, although glutamine excretion appeared to be decreasing as well *(Figure 1).*

Sulfoconjugate excretion for all 3 days of the self-selected diet were not statistically different from the mean of the 7 days of the semisynthetic diet using Helmert transformation for repeated measures analysis *(Table 2).* Although there was no significant difference among the excretion levels of the 3 days of the self-selected dietary period, sulfoconjugate excretion for each day of the semisynthetic diet was significantly different ($P < 0.05$) from subsequent days within the semisynthetic dietary period. Although there was no statistical difference between the two dietary periods, the significant difference in excretion levels that were observed during the semisynthetic dietary period would indicate that there were dynamic changes, i.e., bodily adaptations to the new diet, etc., that occurred during the dietary period.

After day 3 of the semisynthetic diet, sulfoconjugate excretion increased, and the increase continued until the end of

Table 3 Comparison of 3- and 7-day mean urinary conjugate levels in 18 adult males consuming a self-selected and subsequent semisynthetic diet*

Diet	Conjugates (mmol/24hr)†		
	Glucuronides	Sulfoconjugates	Mercapturates
Self-selected (3 day)	2.93 ± 0.77	5.28 ± 2.54	0.27 ± 0.11‡
Semisynthetic (7 day)	3.21 ± 0.29	5.44 ± 1.56	0.14 ± 0.02

*Enrich with Fiber, Ross Laboratories, Columbus, OH USA.
†Mean ± SD.
‡Significantly different from the semisynthetic diet $P < 0.0001$.

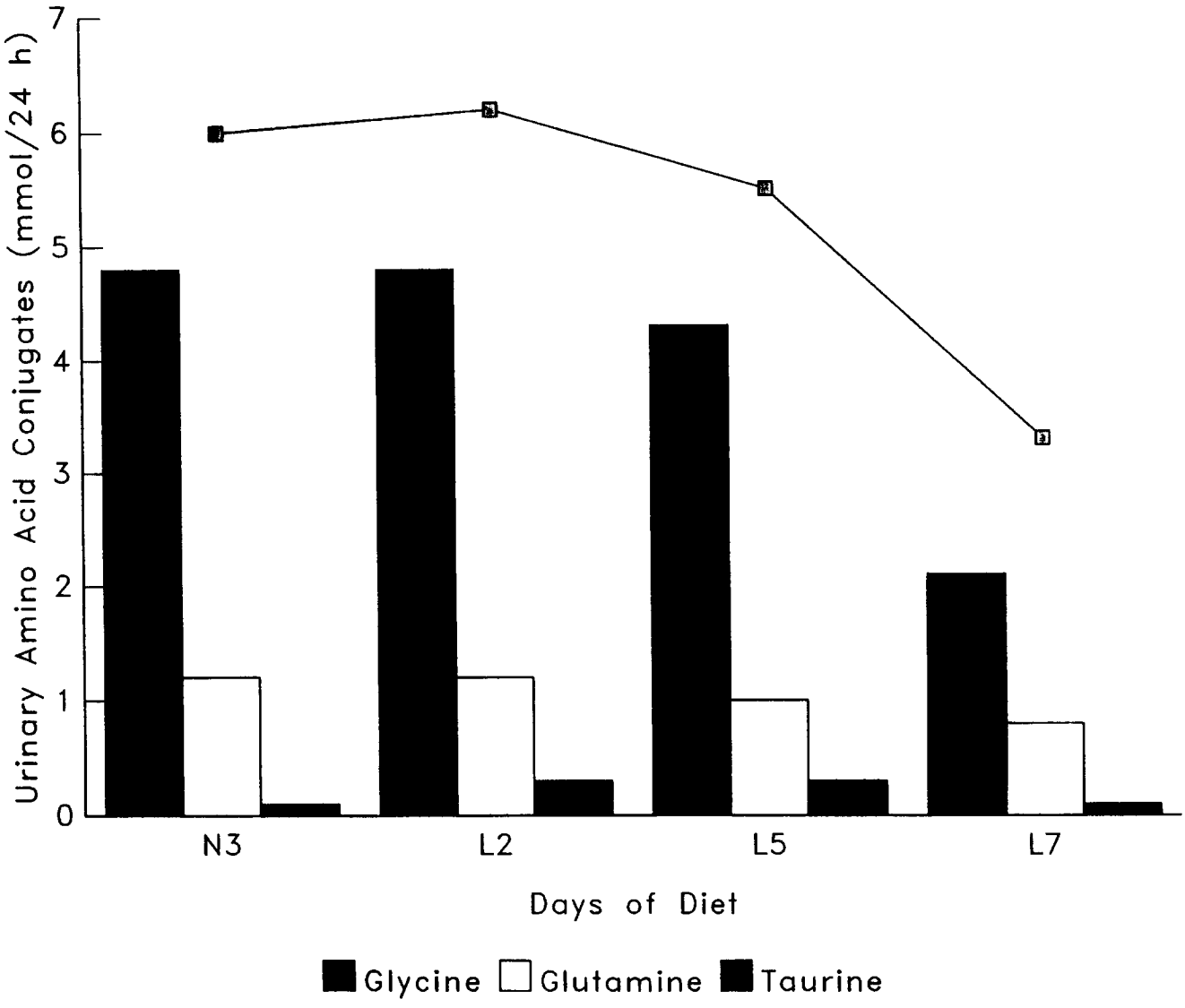

Figure 1 Mean excretion of glycine ■, glutamine □, and taurine ■ conjugates of 18 adult male subjects consuming a self-selected diet for 3 days and a semisynthetic diet (Enrich with Fiber, Ross Laboratories, Columbus, OH USA) for 7 days. N3: third day of self-selected diet; L2, L5, L7: second, fifth, and seventh days of semisynthetic diet; ▒——▒: total amino acid conjugates.

the dietary period *(Table 2)*. The increase in sulfoconjugate excretion may have been due, in part, to certain substances that were in the semisynthetic diet that were preferentially conjugated via sulfation. The semisynthetic diet product used was flavored with vanilla, which is a 3-ethoxy-4-hydroxybenzaldehyde. Mamer et al.[25] found metabolites of this compound in patients who had been given synthetic diets flavored with vanilla. Because of its structure, the benzaldehyde is more likely to be conjugated via sulfation or glucuronidation rather than with glutathione. This would explain the lack of increase in mercapturate excretion while on the semisynthetic diet. A sulfoconjugate increase rather than glucuronide increase may have been due to the favorable competition of sulfation with glucuronidation because of the low K_m value of the sulfotransferases. Why there was a lagtime of 3 days before sulfoconjugates began to increase is not known.

Glucuronide excretion levels seemed to be the least affected by the change in diet. Although there was a slight increase in the excretion of these conjugates, the increase was not significant. This insensitivity to diet change may have been due to the fact that glucuronidation is possibly utilized more for the metabolism of endogenous compounds rather than exogenous compounds. Glucuronide excretion levels may also have been masked by the high excretion of the sulfoconjugates. Sulfoconjugation and glucuronidation are competing pathways for many of the same xenobiotics. Because of the low K_m of the sulfotransferases, the enzymes that catalyze the sulfoconjugation of xenobiotics, sulfoconjugation is usually the more predominant of the two pathways. Glucuronidation becomes the dominant conjugation reaction when xenobiotic levels go up or when sulfate is limiting in the diet.[26,27] On the semisynthetic diet, xenobiotic concentration was theoretically low and there was sufficient sulfate

in the diet. The possibility also exists that the municipal water supplied to the subjects for drinking purposes may have been an additional, extraneous, source of sulfate. Hindmarsh et al.[28] found that increased sulfate levels in drinking water increased the sulfate concentration in serum, further suggesting that sulfate was not limiting in the present study.

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

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