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The Effect of Dietary Sulfur on: I. Glutathione S-Transferase Activity in Rat Lung and Liver; and II. The Hepatic Metabolism and Urinary Excretion of Acetaminophen in Adult Male Rats

Diane Helen Morris
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To the Graduate Council:

I am submitting herewith a dissertation written by Diane Helen Morris entitled "The Effect of Dietary Sulfur on: I. Glutathione S-Transferase Activity in Rat Lung and Liver; and II. The Hepatic Metabolism and Urinary Excretion of Acetaminophen in Adult Male Rats." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

John T. Smith, Major Professor

We have read this dissertation and recommend its acceptance:

Roy E. Beauchene, Robert Feinberg, Daniel Hubbard

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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THE EFFECT OF DIETARY SULFUR ON: I. GLUTATHIONE S-TRANSFERASE ACTIVITY IN RAT LUNG AND LIVER; AND II. THE HEPATIC METABOLISM AND URINARY EXCRETION OF ACETAMINOPHEN IN ADULT MALE RATS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Diane Helen Morris

June 1982

In Memory of

Dr. Mary Rose Gram

who inspired me to begin this work

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ABSTRACT

In Part I, the effect of dietary sulfur on glutathione S-transferase (GSH S-T) activity was evaluated in male Sprague-Dawley rats. The animals were fed diets containing inorganic sulfate ($SO_4^=$) at the levels of 0.0072%, 0.027%, or 0.427%. At each level of $SO_4^=$, there was either no organic sulfur supplementation or supplementation with cysteine or methionine.

There was no effect of dietary $SO_4^=$ on GSH S-T activity in lung and liver supernatants when the group means were analyzed by the method of planned comparisons. There were significant interaction effects between treatments.

Supplementation of diets with organic sulfur reduced GSH S-T activity in both tissues. GSH S-T activity was greater in lung supernatants when rats were fed diets containing cysteine versus methionine. The opposite effect was observed in liver supernatants.

In Part II, the effect of dietary sulfur on the hepatic metabolism and urinary excretion of acetaminophen (APAP) in rats was examined. Male Sprague-Dawley rats were fed diets identical to those used in Part I. Twenty-four hours prior to sacrifice, rats received an injection (i.p.) of APAP (300 mg/kg) in saline. Control animals received an injection of saline alone.

In the liver administration of APAP increased tissue glutathione (GSH) concentration but did not affect the activities of GSH S-T, sulfotransferase (ST), and UDP-glucuronyltransferase (UDP-GT). Dietary $SO_4^=$ had no effect on hepatic enzyme activities or GSH concentration.

Hepatic GSH S-T activity was decreased and GSH concentration was increased in rats fed diets supplemented with organic sulfur.

In the urine, the excretion of APAP sulfate was unaffected by either the level of dietary $SO_4^=$ or the presence or type of organic sulfur supplementation. The excretion of APAP glucuronide, free APAP, and APAP mercapturic acid was greatest at the lowest (0.0072%) level of dietary $SO_4^=$ and tended to decrease as the level of dietary $SO_4^=$ increased. Supplementation of diets with organic sulfur reduced the excretion of APAP glucuronide and APAP mercapturic acid. Only the excretion of APAP glucuronide was affected by the type of organic sulfur supplementation.

This study provides evidence that xenobiotic metabolism in rats is affected by dietary sulfur.

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CHAPTER I

INTRODUCTION

The metabolism of drugs and other foreign compounds generally occurs in two phases. In Phase I, the foreign compound or xenobiotic undergoes oxidation, reduction, dehalogenation, oxygenation, or desulfuration to form a more hydrophilic product. This reaction is catalyzed by the mixed function oxidase (MFO) system located in the endoplasmic reticulum of the cell and usually results in detoxication of a harmful compound (1). In Phase II, conjugation of the metabolite produced by the Phase I MFO system occurs. The xenobiotic metabolite is conjugated with a small molecule such as glucuronic acid, glucose, glycine, glutathione, glutamine, acetic acid, or sulfate to produce a more polar, hydrophilic compound that is readily excreted (2).

Most research examining the effect of diet on xenobiotic metabolism has focused on the Phase I MFO system. Studies have shown that the activities of these microsomal enzymes are sensitive to dietary manipulations. In particular, the level of intake of protein, carbohydrate, lipid, zinc, iron, and vitamins A and C may affect the ability of the MFO enzymes to metabolize xenobiotic compounds in laboratory animals (3). In this laboratory, Acuff and Smith (4) have shown that the tissue level of cytochrome P-450 is altered in rats fed diets containing different levels of inorganic sulfate.

The response of the Phase II or conjugating enzymes involved in xenobiotic detoxication to dietary manipulations remains largely uninvestigated. In particular, very little is known about the effect

of nutritional status on the activity of the glutathione S-transferases. In one diet-related study, Sikic et al. (5) examined the effect of ascorbic acid deficiency on both liver microsomal enzymes and the glutathione S-transferases in guinea pigs. The activities of both enzyme species were decreased in the scorbutic liver. Benson et al. (6) examined the effect of dietary anti-oxidants on glutathione S-transferase activity in the livers of female CD-1 mice and male Sprague-Dawley rats. The administration of BHA [2(3)-tert-butyl-4-hydroxyanisole] and ethoxyquin dramatically increased the hepatic activity of the glutathione S-transferases in both mice and rats with 1,2-dichloro-4-nitrobenzene, 1-chloro-2,4-dinitrobenzene, p-nitrobenzylchloride, and Δ^5-androstene-3,17-dione as second substrates. The increase in glutathione S-transferase activity was considerably larger in the mouse (4-10 fold) than in the rat.

The present study measured glutathione S-transferase activity in rat lung and liver in order to assess the effect of varying dietary levels of inorganic sulfate on xenobiotic metabolism. In addition, the question of whether enzyme activity is affected by dietary supplementation with organic sulfur was addressed. Since the level of cytochrome P-450 is altered by dietary sulfur in the rat, it is conceivable that glutathione S-transferase activity may also be affected by dietary sulfur manipulations in this laboratory animal.

CHAPTER II

REVIEW OF LITERATURE

Importance of Dietary Sulfur

Compounds containing sulfur are ubiquitous in nature and are required by micro-organisms, plants, and animals for growth and development (7). Included among the significant cellular sulfur-containing compounds are Coenzyme A, the vitamins thiamin, lipoic acid, and biotin, in addition to the amino acids methionine, cysteine, cystine, homocysteine, and cystathionine. Other important cellular constituents containing sulfur include glutathione, S-adenosyl-methionine, taurine, and inorganic sulfate (8).

In the animal kingdom, there are two dietary sources of sulfur--organic and inorganic. In animals, the need for organic sulfur is met primarily by the coenzymes and the amino acids methionine, cysteine, and cystine (9). A need for dietary inorganic sulfate by humans has not been clearly established (10), although inorganic sulfate has been shown to be an important nutrient for the rat (11).

Following the identification of methionine as an essential dietary nutrient, efforts were made to determine the function of this amino acid and to identify the factors that regulate its metabolism. It has been shown that methionine is incorporated into proteins and serves as a precursor in the formation of cysteine, homocysteine, cystine, and taurine (9). Methionine is also converted to S-adenosyl-methionine (SAM) in a reaction involving

adenosine-5'-triphosphate (ATP) and functions in this capacity as the primary methyl group donor in mammalian tissues. A number of important compounds are methylated by SAM, including histamine, acetylserotonin, plant phytosterols, and phosphatidylethanolamine (12). An extensive listing of endogenous and exogenous compounds that derive their methyl groups from SAM is given by Baker (8).

Although an early study in the area of sulfur nutrition described cystine as an indispensible dietary constituent for the rat (13), Jackson and Block (14) later established methionine as an essential amino acid, with dietary cystine supplementation becoming important only when dietary methionine levels are reduced to a sub-optimal point (15). Since the presence of cystine in the diet reduces the need for methionine, cystine is said to "spare" methionine (9). Indeed, Finkelstein and Mudd (16) investigated the methionine-sparing effect of cystine and concluded that in rats fed a diet low in methionine and supplemented with cystine, cystine prevented the irreversible conversion of methionine to cysteine and allowed methionine to either remain in the remethylation pathway or be utilized for protein synthesis.

In view of the ability of cystine to reduce methionine catabolism, the replacement value of cystine for methionine has been estimated. Values differ among animal species, but it has been shown that cystine can supply from 17 to 68% of the total sulfur amino acid requirement in rats (8, 15). In humans, cystine can replace approximately 80% of the methionine requirement (17).

The function of dietary inorganic sulfate has also been studied. There is evidence that inorganic sulfate is incorporated into sulfomucopolysaccharides (9), sulfolipids, heparin, and fibrinogen (8). It is also used in the biosynthesis of taurine and chondroitin (18) and is incorporated into cysteine but not methionine. Indeed, with the observation that sulfate could be incorporated into cysteine, it was determined that from 7 to 9% of the dietary sulfur amino acid requirement can be met by inorganic sulfate in chicks (19).

In this laboratory, the metabolic role of dietary inorganic sulfate has been investigated extensively. It has been shown that the addition of inorganic sulfate to the diets of rats improved feed efficiency ratios (20), increased the incorporation of $^{35}SO_4$ into rib cartilage mucopolysaccharides (21), reduced the urinary excretion of taurine (22), and altered the glycocholic:taurocholic acid ratio in bile extracted from sections of rat intestines (23). Other work demonstrated an alteration in collagen metabolism (24) and a reduction in aorta breaking strength (25) in rats fed diets low in inorganic sulfate.

The demonstration that dietary inorganic sulfate is an important nutritional factor for the rat led Smith (26) to determine the optimal dietary level of inorganic sulfate required by this mammal. Test diets were fed to adult male Long-Evans rats for 7 days. At the end of this period, either 1-^{14}C-methionine or U-^{14}C-cysteine was administered and the expired $^{14}CO_2$ was collected and measured. Since $^{14}CO_2$ expiration from labelled methionine was approximately three times greater than that from cysteine, methionine was selected for

determining the optimal dietary level of inorganic sulfate. Using five levels of inorganic sulfate (0.01, 0.02, 0.03, 0.04, 0.05%), Smith showed a reduction in $^{14}CO_2$ expiration when rats were fed diets containing 0.02% inorganic sulfate. Based on these data, Smith concluded that the optimal dietary level of inorganic sulfate is 0.02% for the rat.

A role for inorganic sulfate in the regulation of a number of metabolic processes has been suggested by Smith. An increased incorporation of the carbon skeleton of U-^{14}C-cysteine into glycogen in the livers of rats fed diets containing the optimal level of inorganic sulfate (0.02%) has been demonstrated. In addition, the concentration of glutathione is greatest in the liver but not kidneys of rats fed diets containing 0.02% inorganic sulfate (11). Lipogenesis is also sensitive to the level of dietary inorganic sulfate as evidenced by alterations in the activities of acetyl-CoA carboxylase, citrate cleavage enzyme, and malic enzyme (27).

Characterization of the Glutathione S-Transferases

The glutathione S-transferases (E. C. 2.5.1.18) constitute a family of enzymes involved both in the biotransformation of drugs and other foreign compounds (28) and in the transport and intracellular storage of endogenous compounds such as bilirubin (29). These enzymes are widely distributed in biological materials and have been studied extensively in the rat and humans (30). In the rat, glutathione S-transferase activity has been detected in liver, lung, kidney, spleen, small intestine, brain, heart, testis, ovary, and the adrenals (31).

Purification of the glutathione S-transferases (GSH S-T) from the supernatant fractions of rat liver and kidney homogenates indicates an enzyme molecular weight of approximately 45,000 - 50,000 daltons (32, 33). Each enzyme species consists of two of three possible subunits with the following molecular weights: subunit a - 22,000 daltons; subunit b - 23,500 daltons; subunit c - 24,000 daltons (34). Scully and Mantle (35) have postulated that the dimers are produced from a common homodimer (cc) via a proteolytic mechanism.

Early studies described the glutathione S-transferases as cytosolic enzymes (32), but there is evidence that they are also found in the microsomal fraction of rat liver homogenates (36, 37). Friedberg et al. (38) detected GSH S-T activity in a number of cell organelles, including the rough and smooth reticuli, microsomes, nucleus, plasma membrane, and the Golgi apparatus.

In their roles as Phase II enzymes in the biotransformation of xenobiotics, the glutathione S-transferases catalyze the conjugation of reduced glutathione with a variety of foreign compounds having an electrophilic center. This is the first step in mercapturic acid formation (39). Figure 1 outlines the formation of a mercapturic acid with glutathione and 1-chloro-2,4-dinitrobenzene as substrates. Mercapturic acids are N-acetyl-L-cysteine derivatives that function as the biliary and urinary excretion products of potentially harmful electrophilic compounds (28, 40). The tripeptide glutathione has been identified as the source of the mercapturic acid cysteine moiety (41).

In addition to their catalytic properties, the glutathione S-transferases function as binding proteins (42). Reyes et al. (43)

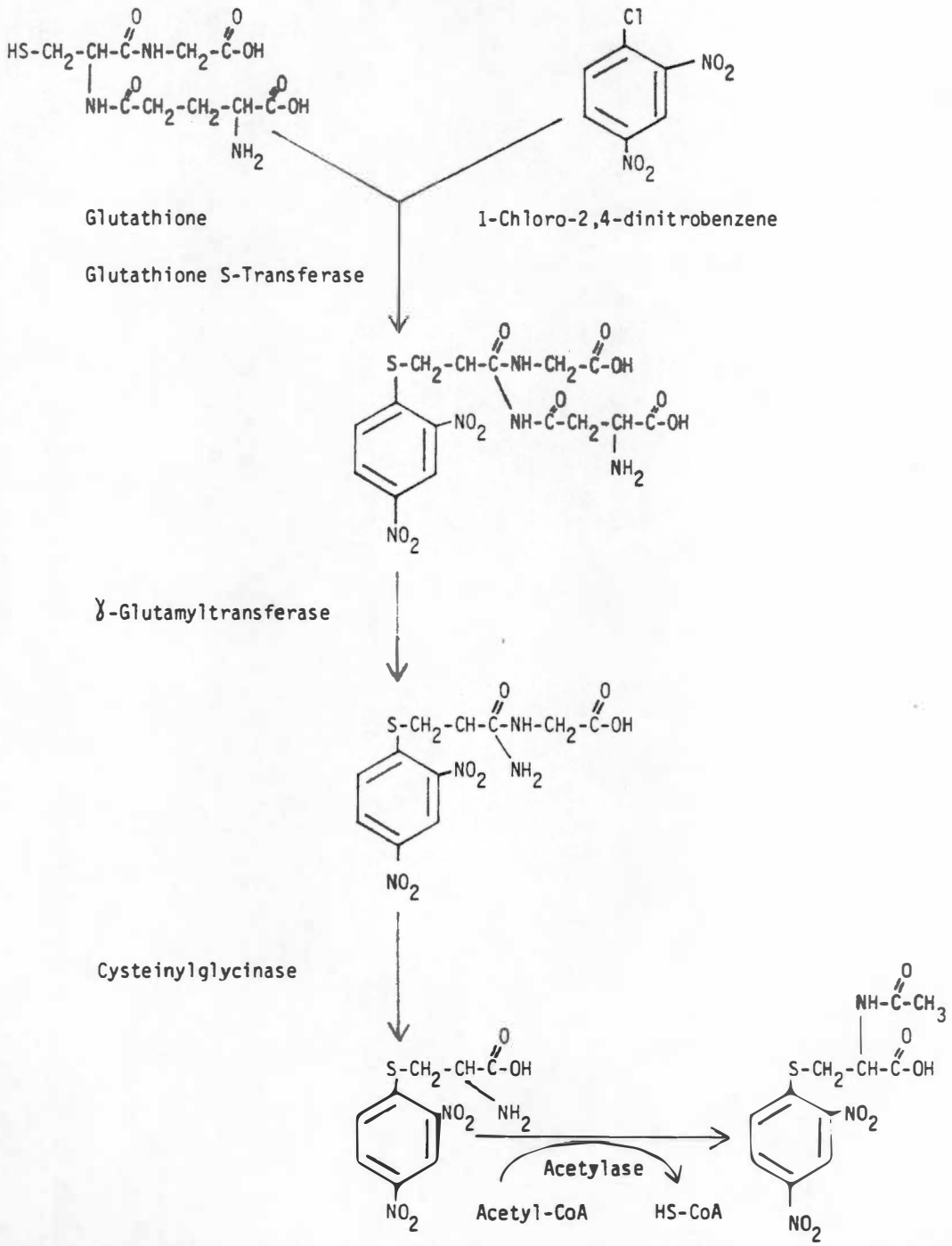


Figure 1. The biosynthesis of a mercapturic acid from the substrates glutathione and 1-chloro-2,4-dinitrobenzene, as adapted from (39).

and Levi et al. (44) examined the ability of two cytoplasmic proteins, Y and Z, to bind the organic anion bilirubin and sulfobromophthalein in rat liver. Y protein, in particular, has been studied intensively and was previously designated azo-dye binding protein or corticosteriod binder I (39). It is widely recognized that this cytoplasmic protein is identical to glutathione S-transferase B or ligandin. Ligandin is one member of a family of organic anion-binding proteins now known to be synonymous with the glutathione S-transferases (30, 33).

Litwack et al. (45) and other researchers (43, 44, 46, 47) report that the ligandin proteins will bind non-covalently a variety of compounds, including steroids, bilirubin, sulfobromophthalein, azo-dyes, and carcinogenic hydrocarbons. Kamisaka et al. (42) describe the ligandins as intracellular binding proteins. They postulate that the human glutathione S-transferases may serve as intracellular storage sites for bilirubin in particular and as a transport mechanism for a variety of ligands from plasma into the liver cell.

Bhargava et al. (47) investigated both bilirubin binding and glutathione S-transferase activity and report that these separate functions occur independently of each other. According to these researchers, two bilirubin binding sites exist on the ligandin molecule--a primary site requiring cysteine residues and a secondary site in which sulfhydryl groups are not essential for catalytic or binding activity.

The kinetic mechanism of the GSH S-T catalyzed reaction has been studied by Pabst et al. (48). Using a variety of substrates, these researchers determined that at physiological concentrations of reduced glutathione (3-10 mM in rat liver), the kinetic mechanism follows an ordered sequential pathway with glutathione binding initially to the enzyme molecule. At low glutathione concentrations, a ping-pong kinetic mechanism is evident in which the second substrate binds first. This theory has not been accepted by Jakobson et al. (49) who propose an alternate kinetic mechanism involving the random binding of substrates to the enzyme molecule and product inhibition of enzyme activity.

It was orginally thought that the glutathione S-transferases were specific for glutathione as a primary substrate (39), but Habig et al. (50) showed that an equimolar concentration of homoglutathione (γ-glutamyl-cysteinyl-β-alanine) was as active as glutathione in GSH S-T catalyzed conjugation reactions. Regarding the second substrate, the glutathione S-transferases exhibit considerable variability in substrate specificity. Initially, these enzymes were named according to the primary type of second substrate conjugated--e.g., glutathione S-aryl-, alkyl-, or epoxide transferase (32, 33, 51, 52). They are now designated AA, A, B, C, D, E, and M based on their elution order from carboxymethyl cellulose columns (39).

Induction of the glutathione S-transferases by the administration of phenobarbitol and polycyclic aromatic hydrocarbons has been demonstrated (39). Clifton and Kaplowitz (53) studied the effect of oral administration of phenobarbitol, 3,4-benzo(a)pyrene, and

3-methylcholanthrene on GSH S-T activity in rat liver, intestine, and kidney. All three inducing agents significantly increased enzyme activity in rat liver with the following substrates: 3,4-dichloronitrobenzene, p-nitrobenzyl chloride, and ethacrynic acid. GSH S-T activity in rat liver was not significantly increased by 3,4-benzo(a)pyrene with the substrate 1-chloro-2,4-dinitrobenzene. Enzyme activity in the rat intestine and kidney was variable in response to the inducing agents.

An early study by Darby and Grundy (54) reported a small increase in rat liver GSH S-aryltransferase activity following phenobarbitone treatment. Sulfobromophthalein, 1,2-dichloro-4-nitrobenzene, and 1-chloro-2,4-dinitrobenzene were used as substrates. A sex difference was observed; males showed a significant increase in GSH S-T conjugation with 1-chloro-2,4-dinitrobenzene as the second substrate.

CHAPTER III

EXPERIMENTAL PROCEDURE

General Plan

The purpose of this study was to examine the effect of dietary sulfur on glutathione S-transferase activity in rat lung and liver. The experimental plan employed a balanced design so that at each inorganic sulfate level, there was either no organic sulfur supplementation or supplementation with either cysteine or methionine. Figure 2 presents the basic experimental format regarding diet designations and outlines the contrasts made in the statistical analysis of the data (see Statistical Analyses, pages 20-23).

Male Sprague-Dawley rats (300-400 grams) were randomly selected from the rat colony of The University of Tennessee Department of Nutrition and Food Sciences. Rats were housed in stainless steel cages with wire-mesh bottoms. No more than three rats were housed in one cage; cages were segregated by diet. Distilled water was freely available. Diets were fed ad libitum for 21 days.

At the end of the dietary period, the rats were killed by decapitation. Lungs and livers were removed, homogenized, and finally centrifuged to prepare the supernatant fraction. Glutathione S-transferase activity was determined spectrophotometrically using the method of Habig et al. (50). The substrate 1-chloro-2,4-dinitrobenzene was selected because it reacts readily with the glutathione S-transferases to give the product S-(2,4-dinitrophenyl)-glutathione (40, 50). The reaction is shown in

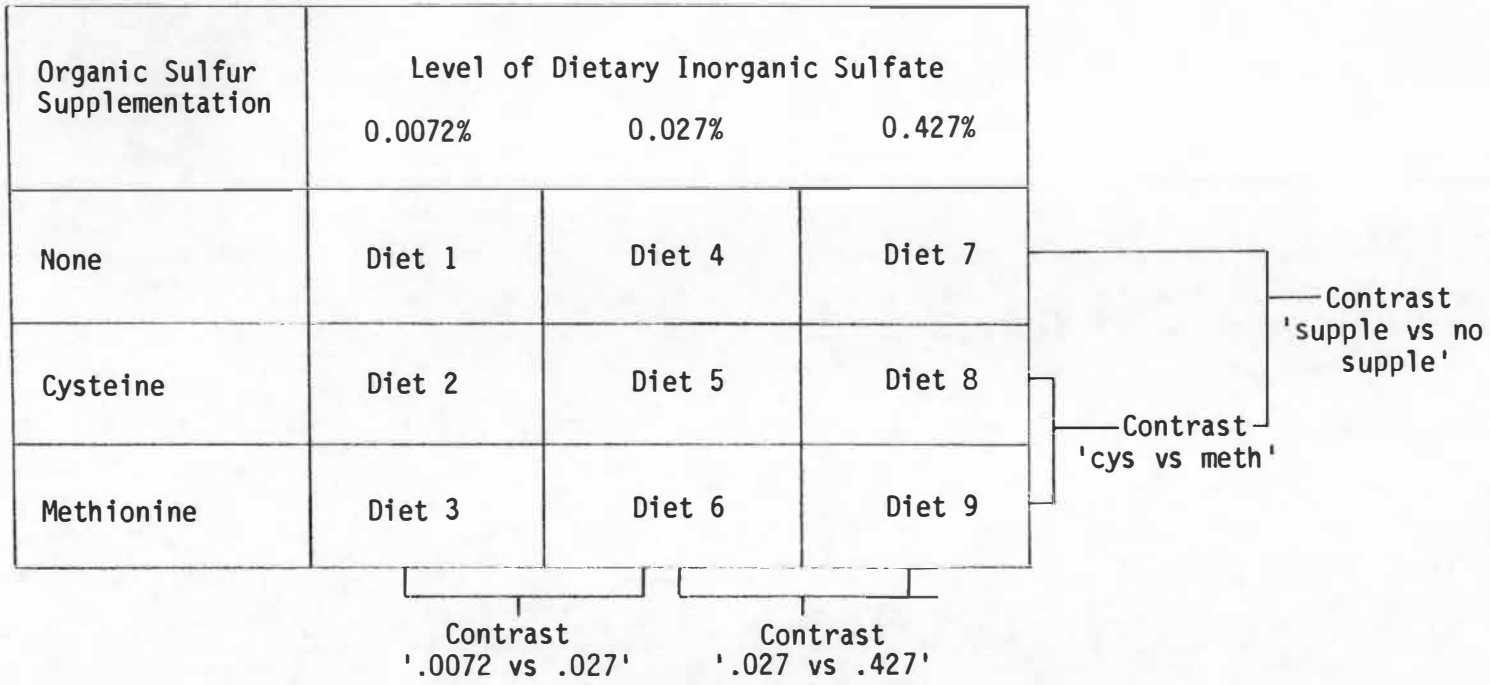


Figure 2. Basic experimental format.

Figure 3. The method of Lowry et al. (55) was used to determine tissue protein concentration with bovine serum albumin as the standard.

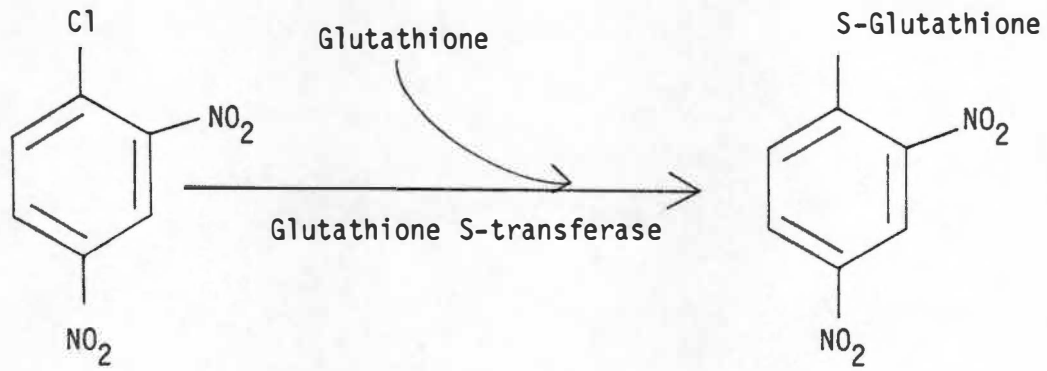
Description of Diets

Semi-purified diets were formulated to maintain general good health in adult rats. Previous work in this laboratory has established a level of 0.02% inorganic sulfate in the diet as the optimal level for the rat; a level of 0.0002% inorganic sulfate in the diet is inadequate in terms of sparing the catabolism of methionine in rats (26). In this experiment, dietary inorganic sulfate levels were 0.0072%, 0.027%, and 0.427%.

The composition of the diets is listed in Tables 1 and 2. Rats were fed the basal diet (Table 1) plus one of the diet formulations listed in Table 2. Diets were fed ad libitum for a period of 21 days. Metabolic alterations have been demonstrated in rats fed diets containing 0.0002%, 0.10%, and 0.42% inorganic sulfate for a period of 17 days (22).

Measurement of Glutathione S-Transferase Activity

At the end of the dietary period, the rats were stunned by a blow to the head and quickly decapitated. Livers and lungs were removed, placed immediately in tared beakers chilled on ice containing about 50 ml of 10 mM TRIS buffer, pH 8.0, and weighed. The livers and lungs were homogenized with a motor-driven Teflon pestle, diluted with the TRIS buffer to make a 10% homogenate, and centrifuged (Model A, Lourdes Instrument Corp., Brooklyn, NY) at 4^{o} for 1.5 hours at 10,000 x g.



1-Chloro-2,4-dinitrobenzene

S-(2,4-dinitrophenyl)-glutathione

Figure 3. Reaction catalyzed by the glutathione S-transferases with the substrates 1-chloro-2,4-dinitrobenzene and glutathione (30, 50).

TABLE 1

Composition of the Basal Diet

Component	g/100 g Diet
Casein[1]	15.00
Cornstarch	30.00
Sucrose	30.00
Vegetable Oil[2]	2.00
Vegetable Shortening[3]	6.00
Vitamin Mixture[4]	2.00
Basal Salt Mixture[5]	1.34
Variable Components	See Table 2

[1]ICN Biochemicals, Inc., Cleveland, Ohio 44128.

[2]Wesson Oil, Hunt-Wesson Foods, Inc., Fullerton, California 92634.

[3]Crisco, Procter and Gamble, Cincinnati, Ohio 45202.

[4]ICN Biochemicals, Inc. Cleveland, Ohio 44128.
Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg vitamin premix): thiamin hydrochloride, 1.0, riboflavin 1.0, niacin 4.5, p-amino-benzoic acid 5.0, calcium pantothenate 3.0, pyridoxine hydrochloride 1.0, ascorbic acid 45.0, inositol 5.0, choline chloride 75.0, menadione 2.25, biotin 0.020, folic acid 0.090, vitamin B_{12} 0.00135, alpha-tocopherol 5.0, vitamin A 9 x 10^5 units, vitamin D 1 x 10^5 units, and sufficient glucose to make 1 kg.

[5]ICN Biochemicals, Inc. Cleveland, Ohio 44128.
Hubbell, Mendel & Wakeman Salt Mixture provided the following (%): calcium carbonate 54.3, magnesium carbonate 2.5, magnesium sulfate·$7H_2O$ 1.6, sodium chloride 6.9, potassium chloride 11.2, potassium phosphate (monobasic) 21.2, ferric phosphate 2.05, potassium iodide 0.008, magnesium sulfate·H_2O 0.035, sodium fluoride 0.1, aluminum potassium sulfate 0.017, copper sulfate·$5H_2O$ 0.09.

TABLE 2

Variations of the Basal Diet

Diet	Dietary Components					
	Inorganic $SO_4^=$	$CaSO_4 \cdot 2H_2O$	$CaCO_3$	DL-Methionine	Cysteine Free Base[1]	Non-Nutritive Bulk[2]
	%	g/100 g Diet				
1	0.0072	0.0	1.34	0.0	0.0	12.32
2	0.0072	0.0	1.34	0.0	0.53	11.79
3	0.0072	0.0	1.34	0.65	0.0	11.67
4	0.027	0.04	1.32	0.0	0.0	12.30
5	0.027	0.04	1.32	0.0	0.50	11.80
6	0.027	0.04	1.32	0.62	0.0	11.68
7	0.427	0.75	0.91	0.0	0.0	12.00
8	0.427	0.75	0.91	0.0	0.50	11.50
9	0.427	0.75	0.91	0.62	0.0	11.38

[1]ICN Biochemicals, Inc. Cleveland, Ohio 44128.

[2]Alphacel, ICN Biochemicals, Inc. Cleveland, Ohio 44128.

Following centrifugation, floating lipid was removed and the supernatant decanted into chilled beakers. For the liver preparations, a 1:25 dilution of the supernatant was made (1 ml supernatant to 24 ml 10 mM TRIS buffer, pH 8.0). The lung supernatant was diluted 5-fold with 10 mM TRIS buffer, pH 8.0. One ml of the diluted supernatant was combined with 3 ml of a reaction mixture containing 25.3 mg 1-chloro-2,4-dinitrobenzene (1.0 mM), 5 ml pure ethanol, and 38.5 mg reduced glutathione (1.0 mM) in a .141 M potassium phosphate buffer, pH 6.5. The rate of the enzyme-catalyzed reaction was measured spectrophotometrically (Model DU, Beckman Instruments, Inc., Fullerton, CA 92634) by following the change in absorbance at 340 nm at one minute intervals for 5 minutes. This method is outlined by Habig et al. (50). All samples were run in duplicate.

Measurement of Tissue Protein

The method of Lowry et al. (55) was used to determine tissue protein concentration with bovine serum albumin as a standard (200 μg/ml .5 N NaOH). The liver supernatants were diluted 2-fold with 1 N NaOH. The lung supernatants were diluted 2-fold with 1 N NaOH and then 3-fold with .5 N NaOH. One ml of the final diluted liver and lung samples was added to 5 ml of reagent A (1 ml 2.7% $NaK \cdot C_4H_4O_6 \cdot 4H_2$, 1 ml 1% $CuSO_4 \cdot 5H_2$, and 100 ml 2% Na_2CO_3), mixed with a Vortex-Genie, and allowed to stand for 10 minutes. After 10 minutes, 0.5 ml of the Folin reagent was added and the sample mixed immediately. After the color had been allowed to develop for 30 minutes, the absorbance of

the samples was read at 750 nm. Duplicate samples for determining protein concentration were analyzed. The following equation was used to calculate protein concentration:

$$\frac{\text{Absorbance (Sample)}}{\text{Absorbance (Std)}} \times \text{Conc. (Std)} \times \text{Dilution Factor} = \text{mg protein}$$

Statistical Analyses

The data were analyzed by the method of planned comparisons. The use of this statistical method allowed the researcher to answer a restricted number of specific questions. That is, the researcher determined à priori that these questions were the most important ones in terms of the purpose and design of the experiment. In general, the comparisons selected were designed to be independent in contrasting means or groups of means and ensured that non-overlapping, non-redundant bits of information were obtained from the data (56).

A sample comparison is defined by Hays (56) as follows:

$$\hat{\psi} = c_1 M_1 + \ldots + c_J M_J = \sum_j c_j M_j$$

where $\hat{\psi}$ (psi hat) equals a weighted sum of numbers, c_j is a weighted sum for treatment j, and M_j is the sample mean for treatment j. When the sum of the weights is zero, the comparison is called a contrast.

In this study, four questions were considered important in view of the constraint of being allowed (J minus 2) contrasts where J = number of treatments. The specific contrasts used to analyze the data from this study are outlined in Figure 2. The four central questions were as follows:

1. Does enzyme activity differ in rats fed diets containing

0.0072% inorganic sulfate versus those fed 0.027% inorganic sulfate (Contrast '.0072 vs .027')?

2. Does enzyme activity differ in rats fed diets containing 0.027% inorganic sulfate versus those fed 0.427% inorganic sulfate (Contrast '.027 vs .427')?
3. Does supplementation with organic sulfur (cysteine + methionine) affect enzyme activity (Contrast 'supple vs no supple')?
4. Does the nature of the supplementation (cysteine versus methionine) affect enzyme activity (Contrast 'cys vs meth')?

The General Linear Models (GLM) Procedure of the Statistical Analysis System (SAS) package was used to evaluate the data (57). The SAS computer program used to generate the contrast statements by the GLM Procedure (PROC GLM) command is outlined in Appendix A. Disproportionality in sample size was accounted for by the PROC GLM command in making independent contrasts between treatment means. In evaluating statistical differences an alpha level of 0.05 was selected.

To test for significant differences in glutathione S-transferase activity in rat lung and liver as a result of varying dietary inorganic sulfate levels, two contrasts were made. The '.0072 vs .027' contrast compared the group mean of diets 1, 2, and 3 with the group mean of diets 4, 5, and 6 (see Figure 2, page 14). The '.027 vs .427' contrast compared the group mean of diets 4, 5, and 6 with the group mean of diets 7, 8, and 9. The effect of dietary supplementation with organic sulfur (Contrast 'supple vs no supple')

on GSH S-T activity in rat lung and liver was made by comparing the group mean of diets 1, 4, and 7 with the group mean of diets 2, 3, 5, 6, 8, and 9. Contrast 'cys vs meth' was made by comparing the group mean of diets 2, 5, and 8 with the group mean of diets 3, 6, and 9. The 'cys vs meth' contrast allowed a comparison of the effect of the type of dietary organic sulfur supplementation on GSH S-T activity in rat lung and liver.

Before the data could be analyzed using the method of planned comparisons, the values of enzyme activity for all variables had to be adjusted for diets 1, 3, 4, 6, 8, and 9. This was necessary since it was observed that the reaction mixture used in the enzyme assay in tissue samples of rats fed these diets lost potency over a 5-day period. Therefore, the values for enzyme activity for each variable were plotted by diet and analyzed by analysis of covariance using the SAS package.

Analysis of covariance (ANCOVA) is a type of regression model that can be used to reduce experimental errors (58). In analysis of covariance, an independent quantitative variable is selected and designated the covariable X. The relationship between the covariable X and the dependent variable Y is then examined and used to remove extraneous variation in Y due to X (59). In this study, the covariable X was time (day 1-5); the dependent variable Y was enzyme activity. The ANCOVA program outlined in Appendix B was used to test for interaction effects between X (time) and Y (enzyme activity) for each lung and liver variable. The non-significant F-values for interaction effects for each variable shown in Appendix C indicate

that the regression lines are parallel. The significance of this is that evidence of parallelness among the regression lines indicates that the values of Y (enzyme activity) are not confounded by the covariable X (time). Therefore, statements can be made about the "true" values of Y (60).

In the general ANCOVA model, the values of Y are evaluated at the median value of X (in this study, day 3). However, since the regression lines are parallel, Y values can be inspected at any value of X (60). Therefore, day 1 was selected as the inspection point since the reaction mixture was freshest on this day. The PROC GLM statement of the SAS package was used to generate solutions to normal equations in order to adjust the values of Y (enzyme activity) to day 1. These adjusted values were then analyzed by the method of planned comparisons as discussed previously.

CHAPTER IV

RESULTS

The purpose of this investigation was to determine the effect of dietary sulfur on the activity of the glutathione S-transferases in rat lung and liver. Rats were fed diets containing 0.0072%, 0.027%, or 0.427% inorganic sulfate with either no organic sulfur supplementation or supplementation with cysteine or methionine. The study was designed to answer four basic questions:

1. Does glutathione S-transferase (GSH S-T) activity differ in rats fed 0.0072% versus 0.027% inorganic sulfate (Contrast '.0072 vs .027')?
2. Does GSH S-T activity differ in rats fed diets containing 0.027% versus 0.427% inorganic sulfate (Contrast '.027 vs .427')?
3. Does supplementation with organic sulfur (cysteine + methionine) affect GSH S-T activity in rat lung and liver (Contrast 'supple vs no supple')?
4. Does GSH S-T activity differ in rats fed diets supplemented with cysteine versus those fed diets supplemented with methionine (Contrast 'cys vs meth')?

Effect of Dietary Sulfur on Glutathione S-Transferase Activity in Rat Lung

The differences between group means with respect to dietary inorganic sulfate levels and the presence and type of organic sulfur supplementation were compared by the method of planned comparisons.

The data and analysis of variance summary for enzyme activity expressed as nmoles CDNB conjugated/mg protein/minute are given in Tables 3 and 4. The data and analysis of variance summary for enzyme activity expressed on a wet weight basis are shown in Tables 5 and 6.

In order to evaluate the effect of dietary inorganic sulfate ($SO_4^=$) on GSH S-T activity in rat lung, two contrasts were made: '.0072 vs .027' and '.027 vs .427'. As shown in Tables 4 and 6, dietary inorganic sulfate appeared to have no effect on enzyme activity when the data were analyzed by the method of planned comparisons. This was true whether the data were expressed as either nmoles 1-chloro-2,4-dinitrobenzene (CDNB) conjugated/mg protein/min. or on a wet weight basis (μmoles CDNB conjugated/g lung tissue wet weight). However, even though there were no statistically significant differences between the group means as defined by the contrast statements, the pattern of the effect of dietary inorganic sulfate on GSH S-T activity in lung tissue was similar regardless of how the data were expressed (see Tables 3 and 5): GSH S-T activity was lowest in the lung supernatant of rats fed diets containing the optimal level of $SO_4^=$ (0.027%), intermediate at the 0.0072% $SO_4^=$ level, and highest at the 0.427% $SO_4^=$ level.

Since it has been shown that the activities of the enzymes involved in the trans-sulfuration pathway are sensitive to the dietary levels of cystine and methionine (16), the effect of dietary supplementation with organic sulfur (cysteine + methionine) on GSH S-T activity was evaluated. This was accomplished by using the 'Supple vs No Supple' contrast. The data presented in Table 3 indicate that GSH

TABLE 3

The Effect of Dietary Sulfur on Glutathione S-Transferase Activity in Rat Lung Expressed as nmoles CDNB[1] Conjugated/mg Protein/Minute

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027%	0.427%	
	nmoles CDNB conjugated/mg protein/minute			
None	4.7 ± 1.1[2](5)[3]	3.6 ± 1.1 (5)	10.8 ± 1.1 (5)	6.4 ± 0.6 (15)
Cysteine (0.5 g/100 g diet)	7.4 ± 1.1 (5)	5.6 ± 0.8 (11)	3.5 ± 1.2 (4)	5.5 ± 0.6 (20)
Methionine (0.6 g/100 g diet)	3.5 ± 1.1 (5)	3.7 ± 1.1 (5)	3.4 ± 1.1 (5)	3.5 ± 0.6 (15)
Group Means	5.2 ± 0.6 (15)	4.3 ± 0.6 (21)	5.9 ± 0.7 (14)	

[1]CDNB = 1-Chloro-2,4-dinitrobenzene.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 4

Analysis of Variance Summary for Glutathione S-Transferase Activity in Rat Lung Expressed as nmoles 1-Chloro-2,4-dinitrobenzene Conjugated/mg Protein/Minute

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	20.1684	1.60	0.2141
Supplementation	2	63.1946	5.01	0.0113
Interaction	4	161.8502	6.42	0.0004
Contrasts Between Group Means				
'.0072 vs .027'	1	6.4671	1.03	0.3169
'.027 vs .427'	1	19.7396	3.13	0.0842
'Supple vs No Supple'	1	33.5821	5.33	0.0261
'Cys vs Meth'	1	31.4693	4.99	0.0309

TABLE 5

The Effect of Dietary Sulfur on Glutathione S-Transferase Activity in Rat Lung Expressed as μmoles CDNB[1] Conjugated/g Lung Tissue (Wet Weight)

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027%	0.427%	
	μmoles CDNB conjugated/g lung tissue (wet weight)			
None	.425 ± .119[2](5)[3]	.397 ± .119 (5)	1.004 ± .119 (5)	.608 ± .068 (15)
Cysteine (0.5 g/100 g diet)	.639 ± .119 (5)	.569 ± .080 (11)	.330 ± .132 (4)	.512 ± .065 (20)
Methionine (0.6 g/100 g diet)	.437 ± .119 (5)	.453 ± .119 (5)	.409 ± .119 (5)	.433 ± .068 (15)
Group Means	.500 ± .068 (15)	.473 ± .062 (21)	.581 ± .071 (14)	

[1]CDNB = 1-Chloro-2,4-dinitrobenzene.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 6

Analysis of Variance Summary for Glutathione S-Transferase Activity in Rat Lung Expressed as μmoles 1-Chloro-2,4-dinitrobenzene Conjugated/g Lung Tissue (Wet Weight)

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	9.4974	0.68	0.5106
Supplementation	2	23.2552	1.66	0.2034
Interaction	4	130.9908	4.66	0.0034
Contrasts Between Group Means				
'.0072 vs .027'	1	0.6268	0.09	0.7666
'.027 vs .427'	1	9.2290	1.31	0.2583
'Supple vs No Supple'	1	18.7720	2.67	0.1097
'Cys vs Meth'	1	5.0345	0.72	0.4020

S-T activity in rat lung was significantly increased in rats fed diets lacking organic sulfur when the data were expressed as nmoles CDNB conjugated/mg protein/min. When expressed on a wet weight basis as shown in Table 5, enzyme activity was increased in rats fed diets deficient in organic sulfur.

The 'cys vs meth' contrast tested whether there was a difference in the effect of the amino acids cysteine and methionine on GSH S-T activity in rat lung. The question of whether cysteine and methionine were metabolically equivalent in their effect on GSH S-T activity was considered important since previous data collected in this laboratory had demonstrated differences in cysteine and methionine supplementation (11). Tables 3 and 4 show that when the data were expressed as nmoles CDNB conjugated/mg protein/min., the effect of supplementation of the diets with cysteine or methionine on GSH S-T activity in rat lung was significantly different. Glutathione S-transferase activity was increased in the lung supernatant of rats fed diets supplemented with cysteine. Supplementation of the diets with cysteine increased GSH S-T activity in rat lung when the data were expressed on a wet weight basis (Table 5), although this increase was not statistically significant.

As shown in Tables 4 and 6, there were significant interaction effects between organic sulfur supplementation and the level of inorganic sulfate. The data presented in Tables 3 and 5 indicate that as the level of $SO_4^=$ increased from 0.0072% to 0.427%, enzyme activity was altered as a function of organic sulfur: with no organic sulfur supplementation, enzyme activity increased as the level of $SO_4^=$

increased; with cysteine supplementation, enzyme activity decreased as the level of $SO_4^=$ increased; with methionine supplementation, enzyme activity was unaffected by increasing levels of $SO_4^=$.

Effect of Dietary Sulfur on Glutathione S-Transferase Activity in Rat Liver

The statistical method used to evaluate the effect of dietary sulfur on glutathione S-transferase activity in rat liver was the method of planned comparisons as described previously (see pages 20-23). The data and analysis of variance summary for enzyme activity expressed as μmoles CDNB conjugated/mg protein/minute are presented in Tables 7 and 8. The data and analysis of variance summary for enzyme activity expressed on a wet weight basis are given in Tables 9 and 10.

The data presented in Tables 8 and 10 show that there was no apparent effect of dietary inorganic sulfate on GSH S-T activity in rat liver when the data were analyzed by the method of planned comparisons. This was true whether the data were expressed as μmoles CDNB conjugated/mg protein/min. or on a wet weight basis (μmoles CDNB conjugated/g liver tissue wet weight).

Supplementation of diets with organic sulfur (cysteine + methionine) significantly decreased GSH S-T activity in rat liver when the data were expressed on a wet weight basis (Table 10). When the data were expressed as moles CDNB conjugated/mg protein/min., supplementation of the diets with organic sulfur decreased GSH S-T activity in rat liver, although the reduction was not statistically significant (Table 8).

TABLE 7

The Effect of Dietary Sulfur on Glutathione S-Transferase Activity in Rat Liver Expressed as μmoles CDNB[1] Conjugated/mg Protein/Minute

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027%	0.427%	
	μmoles CDNB conjugated/mg protein/minute			
None	.412 ± .049[2](5)[3]	.375 ± .049 (5)	.287 ± .049 (5)	.358 ± .028 (15)
Cysteine (0.5 g/100 g diet)	.134 ± .049 (5)	.249 ± .035 (10)	.364 ± .049 (5)	.249 ± .026 (20)
Methionine (0.6 g/100 g diet)	.382 ± .049 (5)	.349 ± .049 (5)	.392 ± .049 (5)	.374 ± .028 (15)
Group Means	.309 ± .028 (15)	.325 ± .026 (20)	.348 ± .028 (15)	

[1]CDNB = 1-Chloro-2,4-dinitrobenzene.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 8

Analysis of Variance Summary for Glutathione S-Transferase Activity in Rat Liver Expressed as μmoles 1-Chloro-2,4-dinitrobenzene Conjugated/mg Protein/Minute

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	1.1168	0.47	0.6302
Supplementation	2	15.6697	6.55	0.0034
Interaction	4	16.7971	3.51	0.0148
Contrasts Between Group Means				
'.0072 vs .027'	1	0.1914	0.16	0.6912
'.027 vs .427'	1	0.4329	0.36	0.5507
'Supple vs No Supple'	1	2.1892	1.83	0.1834
'Cys vs Meth'	1	12.8982	10.79	0.0021

TABLE 9

The Effect of Dietary Sulfur on Glutathione S-Transferase Activity in Rat Liver Expressed as μmoles CDNB[1] Conjugated/g Liver Tissue (Wet Weight)

Organic Sulfur	Level of Dietary Inorganic Sulfate			
	0.0072%	0.027%	0.427%	Group Means
	μmoles CDNB conjugated/g liver tissue (wet weight)			
None	31.9 ± 3.4[2](5)[3]	27.2 ± 3.4 (5)	23.3 ± 3.4 (5)	27.5 ± 1.9 (15)
Cysteine (0.5 g/100 g diet)	13.3 ± 3.4 (5)	20.2 ± 2.4 (10)	23.4 ± 3.4 (5)	19.0 ± 1.8 (20)
Methionine (0.6 g/100 g diet)	26.3 ± 3.4 (5)	25.9 ± 3.4 (5)	23.0 ± 3.4 (5)	25.1 ± 1.9 (15)
Group Means	23.8 ± 1.9 (15)	24.4 ± 1.8 (20)	23.2 ± 1.9 (15)	

[1]CDNB = 1-Chloro-2,4-dinitrobenzene.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 10

Analysis of Variance Summary for Glutathione S-Transferase Activity in Rat Liver Expressed as μmoles 1-Chloro-2,4-dinitrobenzene Conjugated/g Liver Tissue (Wet Weight)

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	11.8541	0.10	0.9009
Supplementation	2	640.9572	5.66	0.0068
Interaction	4	474.8776	2.10	0.0987
Contrasts Between Group Means				
'.0072 vs .027'	1	2.9772	0.05	0.8198
'.027 vs .427'	1	11.8207	0.21	0.6502
'Supple vs No Supple'	1	305.0812	5.39	0.0253
'Cys vs Meth'	1	303.2100	5.35	0.0258

Regardless of the manner in which the data were expressed, the nature of the organic sulfur supplementation had a significant effect on GSH S-T activity in rat liver. The data presented in Tables 7 and 9 indicate that GSH S-T activity was increased in the liver supernatant of rats fed diets supplemented with methionine.

As with the lung data, there were significant interaction effects between treatments (Table 8) in terms of enzyme activity in the liver. As the level of $SO_4^=$ increased from 0.0072% to 0.427% as shown in Tables 7 and 9, the following changes in enzyme activity were observed: with no organic sulfur supplementation, enzyme activity decreased; with cysteine supplementation, enzyme activity increased; with methionine supplementation, enzyme activity remained unchanged.

CHAPTER V

DISCUSSION

The optimal level of dietary inorganic sulfate for the rat is 0.02% as established by Smith (26). He has shown that metabolic alterations in carbohydrate and lipid metabolism occur in rat tissues at levels of dietary inorganic sulfate other than the optimal level. In addition, he has provided evidence that xenobiotic metabolism may be altered in response to varying levels of dietary inorganic sulfate. For example, an increase in the level of dietary inorganic sulfate from 0.0002% to 0.42% has been shown to reduce the ratio of glucuronide-salicylamide to sulfate-salicylamide excreted in rat urine (11). Also, an increase in the level of dietary inorganic sulfate from 0.0002% to 0.02% with either cysteine or methionine supplementation increased sleep time in rats injected with sodium pentobarbital (4). Since these parameters of xenobiotic metabolism were affected by dietary inorganic sulfate, the response of the Phase II glutathione S-transferases to inorganic sulfate as a dietary constituent was investigated.

In this study, no effect of dietary inorganic sulfate on glutathione S-transferase (GSH S-T) activity was evident in rat lung or liver when the data (group means) were analyzed by the method of planned comparisons. However, an examination of the data for GSH S-T activity in rat lung and liver reveals several trends: as the level of dietary inorganic sulfate increased, enzyme activity increased, decreased, or remained unchanged, depending upon the presence and type

of organic sulfur supplementation. Normally, the strengths of these associations would be tested statistically by regression analysis. However, the regression model was not considered appropriate for these data because the levels of dietary inorganic sulfate used did not represent consistent increments in value. If the levels of inorganic sulfate in the diets had been 0.0001%, 0.001%, 0.01%, 0.1%, and 1.0%, for example, the use of regression analysis would have been justified (58).

Several investigations in this laboratory have demonstrated that the supplementation of diets with organic sulfur affects a number of metabolic processes in rats (11). The urinary excretion of taurine was increased in rats fed diets containing 0.42% inorganic sulfate ($SO_4^=$) + ^{35}S-cysteine when compared to the taurine excretion level of rats fed diets containing 0.42% $SO_4^=$ without cysteine supplementation (22). The addition of cysteine to the diets of rats fed $SO_4^=$ at the level of 0.42% significantly reduced the number of lung lesions observed following the intratracheal instillation of benzo(a)pyrene (61). The activities of citrate cleavage enzyme and malic enzyme were reduced in the livers of rats fed diets containing 0.42% $SO_4^=$ + cysteine when compared to those fed diets containing only $SO_4^=$ at the level of 0.42%. Similar results were obtained when the 0.42% $SO_4^=$ diet was supplemented with methionine (27).

In this investigation, glutathione S-transferase activity was decreased in the lungs and liver of rats fed diets supplemented with organic sulfur. This observed decrease in the in vitro rate of the enzyme-catalyzed reaction may be a reflection of altered in vivo rates

of enzyme synthesis and degradation or modification of enzyme efficiency (62). In general, enzyme activity or efficiency can be rapidly modified in vivo by negative feedback inhibition, post-translational chemical alteration of enzyme conformation, or the action of allosteric effectors and inhibitors (63). The availability of substrate(s) and the removal of product(s) are also controlling factors in regulating in vivo enzyme activity (62).

In vitro, the concentration of enzyme in a tissue sample is estimated by determining its catalytic activity under standard conditions--i.e., fixed pH, temperature, and near-saturating concentrations of substrates and coenzymes (64). In this study, GSH S-T activity was determined by measuring spectrophotometrically the change in absorbance at 340 nm under specific assay conditions (50). The rate of absorbance change was a reflection of the rate of product [S-(2,4-dinitrophenyl)-glutathione] formation. The data indicate that, in general, the rate of in vitro product formation was greater in tissue samples of rats fed diets deficient in organic sulfur than in tissue samples of rats fed diets supplemented with cysteine and methionine.

Several explanations can be offered for the increase in GSH S-T activity seen in homogenates of rats fed diets deficient in organic sulfur. The increase in GSH S-T activity may be a function of enhanced enzyme synthesis or retarded enzyme degradation in vivo (65). In addition, if conversion of an inactive to an active form of the enzyme is required for the expression of enzyme activity, the rate of enzyme activation may be increased under these conditions. An

increase in the rate of formation of active enzyme molecules may result from covalent modification of the enzyme (63) or cooperativity of ligand binding (66). Evidence for the existence of active and inactive forms of the glutathione S-transferases has not been presented in the literature. However, Vander Jagt et al. (67) have reported that bilirubin binding to a secondary non-specific site exhibiting catalytic activity may alter enzyme conformation and affect transferase activity in rat liver.

The observation that methionine can be pulled from the re-methylation pathway to be converted to cysteine (68) suggests that dietary methionine supplementation would produce metabolic effects similar to those seen with dietary cysteine supplementation. Data collected previously in this laboratory (11) and the data presented here indicate that methionine and cysteine are not equivalent in terms of their metabolic effects in rats. This investigation demonstrates a significant difference in glutathione S-transferase activity in the lung and liver supernatant of rats fed diets supplemented with cysteine versus methionine.

In addition, the nature of the effect differs in lung and liver tissue. In lung tissue, GSH S-T activity was greater when the dietary source of organic sulfur was cysteine as opposed to methionine (Table 3, page 26, and Table 5, page 28). In the liver, supplementation of diets with methionine produced a significant increase in GSH S-T activity (Table 7, page 32, and Table 9, page 34). The significance of these tissue differences is difficult to interpret. Finkelstein (68) reported tissue differences in the activities of the enzymes of

the trans-sulfuration pathway in rat tissues. He suggested that isozymes may be responsible for this observation but noted the lack of support for his idea in the literature.

The presence of significant interaction effects between treatments suggests that the effect of dietary inorganic sulfate on GSH S-T activity in rat lung and liver cannot be totally separated from the presence and type of organic sulfur supplementation. As the level of dietary inorganic sulfate increased, enzyme activity was altered in both lung and liver tissues of rats fed diets either lacking organic sulfur or supplemented with cysteine. Since the nature of the effect differed in these two tissues, it can be concluded that rat lung and liver differ in their metabolic response to inorganic sulfate under these conditions. It may be that the capacity of inorganic sulfate to spare cysteine is greater in lung tissue than in liver tissue of rats.

With methionine supplementation, enzyme activity was essentially unaltered as the level of dietary inorganic sulfate increased. This suggests that the mechanism(s) of control of enzyme activity may differ when diets are supplemented with methionine versus cysteine.

CHAPTER VI

SUMMARY

The purpose of this study was to determine the effect of dietary sulfur on glutathione S-transferase (GSH S-T) activity in rat lung and liver. In this investigation, no effect of dietary inorganic sulfate on GSH S-T activity was apparent in either tissue when the data (group means) were analyzed by the method of planned comparisons. However, there were significant interaction effects between treatments, suggesting that the effect of dietary inorganic sulfate on enzyme activity cannot be totally separated from the effect of organic sulfur supplementation. Indeed, GSH S-T activity varied with increasing levels of dietary inorganic sulfate when the diets were either deficient in organic sulfur or supplemented with cysteine. This was true in both tissues, although the nature of the effect differed. With methionine supplementation, GSH S-T activity remained unchanged as the level of inorganic sulfate in the diet increased. The data suggest that inorganic sulfate may function as a regulator of GSH S-T activity when organic sulfur is either limiting or available as cysteine.

Supplementation of diets with organic sulfur (cysteine + methionine) reduced GSH S-T activity in both lung and liver tissue. The increase in GSH S-T activity in rats fed diets deficient in organic sulfur may represent a compensatory response to the metabolic stress of an organic sulfur deficiency.

This study provides additional evidence that methionine and cysteine are not metabolically equivalent in the rat. In lung tissue, GSH S-T activity was increased in rats fed diets supplemented with cysteine; in liver tissue, GSH S-T activity was increased in rats fed diets supplemented with methionine. The data indicate that the two tissues differ in their response to the type of dietary organic sulfur supplementation.

The significance of this study is that it provides evidence that glutathione S-transferase activity is affected by dietary sulfur manipulations. The data presented here suggest that the ability of these Phase II enzymes to conjugate and thereby detoxify xenobiotic compounds may be affected by the sulfur status of the animal.

PART II: THE EFFECT OF DIETARY SULFUR ON THE HEPATIC METABOLISM AND URINARY EXCRETION OF ACETAMINOPHEN IN ADULT MALE RATS

CHAPTER I

INTRODUCTION

Acetaminophen is a widely used analgesic and is an ingredient of many commercial preparations (69). When administered theraputically, acetaminophen is generally recognized as safe (70). However, excessive injestion of acetaminophen can produce hepatotoxicity in both humans and animals (69, 70, 71).

Currently, there is considerable interest in the mechanism of hepatic necrosis following acetaminophen overdose (69). Compounds that may provide protection against acetaminophen-induced hepatotoxicity have been identified and studied. Among the compounds considered for use as adjuvant agents in the treatment of acetaminophen toxicity are N-acetylcysteine (69, 72), methionine (73, 74), cysteine (73), and sulfate (73, 75).

The issue remains, however, of whether and/or how acetaminophen metabolism is altered as a function of the sulfur status of the animal. Conceivably, the availability of cysteine for glutathione biosynthesis and inorganic sulfate for xenobiotic conjugation may affect acetaminophen metabolism and excretion. It has been demonstrated, for example, that hepatic glutathione levels can be altered by varying dietary inorganic sulfate levels (11). This suggests that the sulfur status of the animal may alter glutathione availability for acetaminophen mercapturic acid formation. Glucuronidation of acetaminophen may also be sensitive to dietary sulfur manipulations, since it has been shown that the ratio of

glucuronide-salicylamide to sulfate-salicylamide excreted in rat urine is reduced with an increase in the dietary inorganic sulfate level from 0.0002% to 0.02% (11). In addition, the results of several studies have indicated that alterations in the inorganic sulfate status of the animal affect acetaminophen sulfate formation (73, 75, 76).

In consideration of these issues, this study examined the effect of dietary sulfur on the hepatic metabolism and urinary excretion of acetaminophen in adult male rats. The activities of UDP-glucuronyltransferase, sulfotransferase, and the glutathione S-transferases following acetaminophen administration were measured in rat liver. Hepatic glutathione concentration was also determined. In urine samples, acetaminophen glucuronide, sulfate, mercapturic acid, and free acetaminophen were quantitated by high-performance liquid chromatography.

CHAPTER II

REVIEW OF LITERATURE

Acetaminophen Metabolism

Acetaminophen [4-hydroxyacetanilid, paracetamol (76), N-acetyl-p-aminophenol (77)] is a metabolic product of phenacetin and acetanilid and possesses both analgesic and antipyretic properties. Acetaminophen is classified as a mild analgesic equivalent in efficacy to aspirin (78).

The glucuronide and sulfate conjugates of acetaminophen are the major excretion products in man (79) and the rat (80). The mercapturic acid conjugate constitutes approximately 3 - 4% of the total dose excreted in rats (81). The formation of these conjugates occurs primarily in the liver in reactions catalyzed by the Phase II enzymes of xenobiotic metabolism. The glucuronidation of acetaminophen is catalyzed by the microsomal enzyme UDP-glucuronyltransferase (E. C. 2.4.1.17). The conjugating compound (glucuronic acid) required in this reaction is obtained from uridine diphosphate glucuronic acid (UDP-GA). The reaction is shown in Figure 4. Sulfation of acetaminophen is catalyzed by the cytoplasmic enzyme sulfotransferase (E. C. 2.8.2.1). As shown in Figure 5, this reaction requires PAPS (3'-phosphoadenosine-5'-phosphosulfate) and ATP (2). Formation of the mercapturic acid conjugate of acetaminophen is catalyzed by the glutathione S-transferases (E. C. 2.5.1.18) as shown in Figure 6. The reaction requires reduced glutathione (2, 39) and is

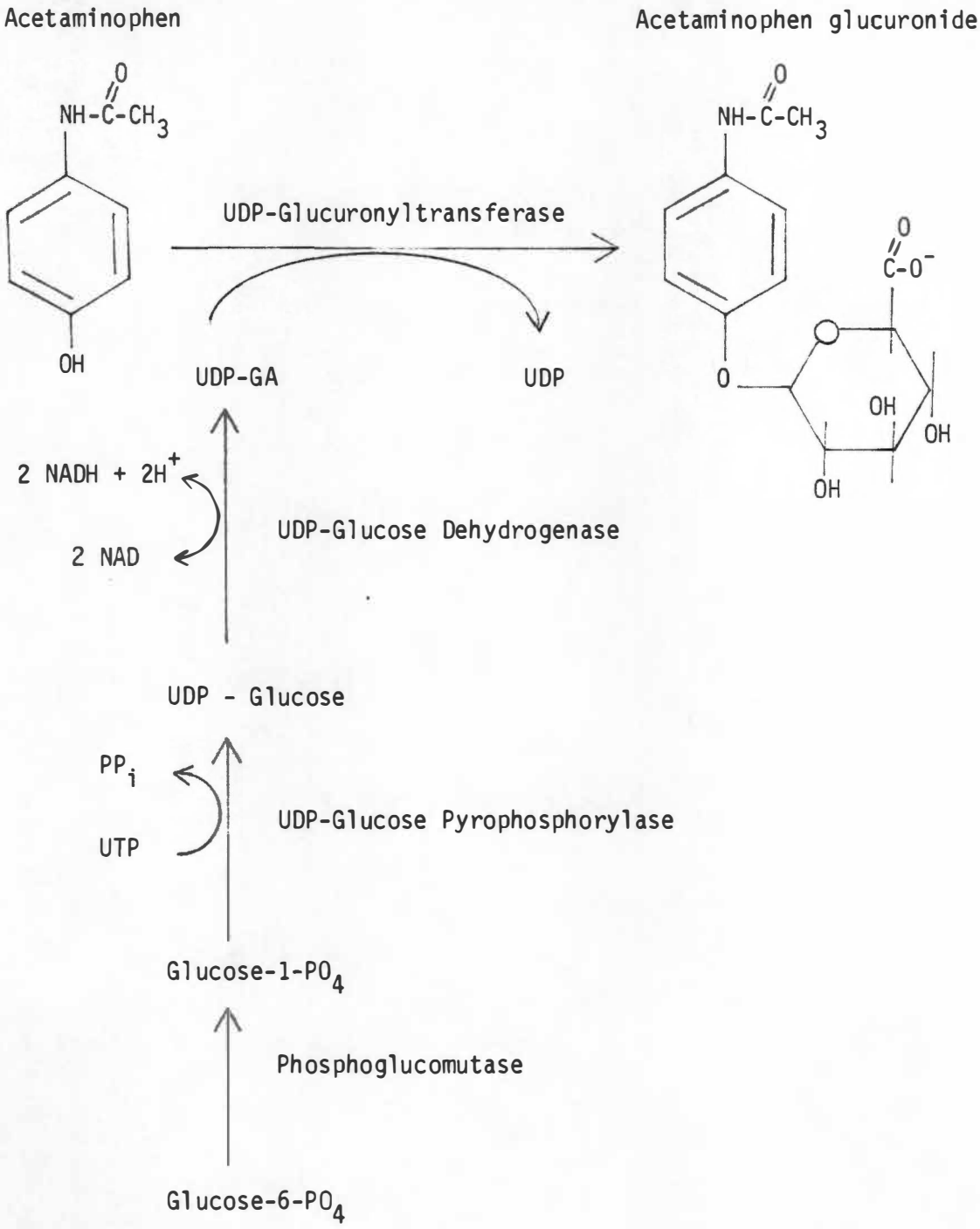


Figure 4. Conversion of acetaminophen to acetaminophen glucuronide by UDP-glucuronyltransferase, including formation of UDP-glucuronic acid (77, 93).

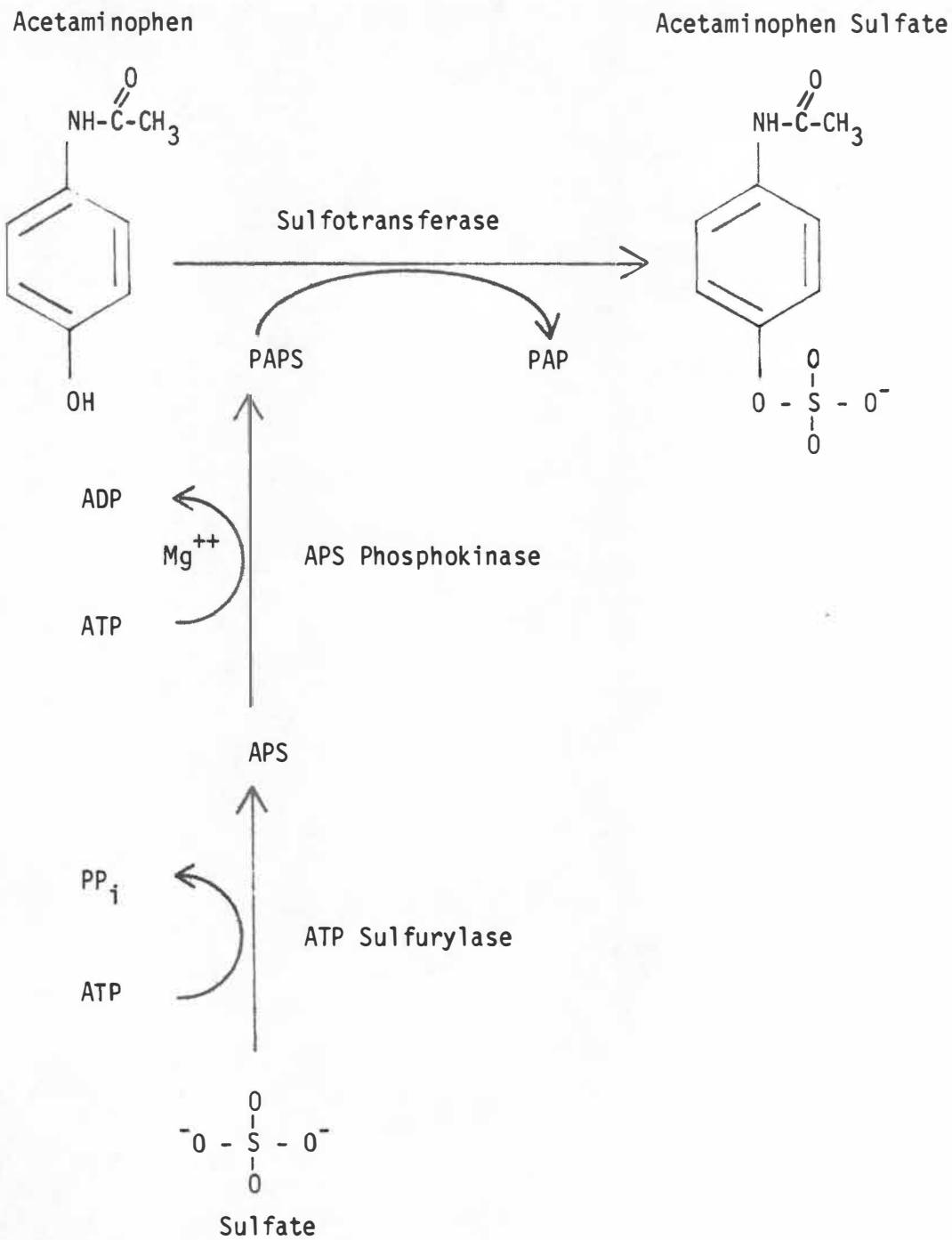


Figure 5. Conversion of acetaminophen to acetaminophen sulfate by sulfotransferase, including the formation of APS (adenosine-5'-phosphosulfate) and PAPS (3'-phosphoadenosine-5'-phosphosulfate) (77, 93).

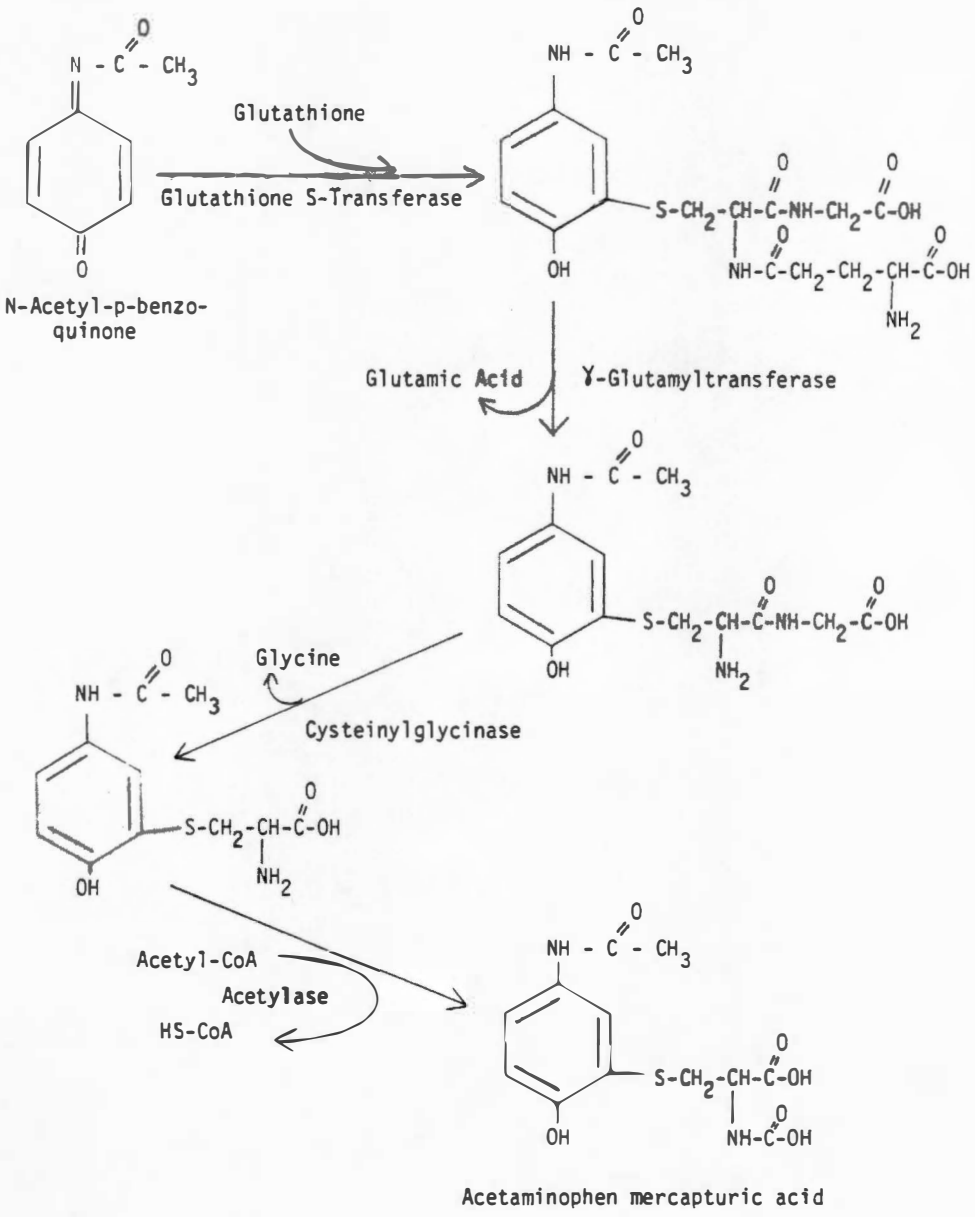


Figure 6. Formation of acetaminophen mercapturic acid from the reactive acetaminophen metabolite N-acetyl-p-benzoquinone (70, 71, 103).

thought to be the primary conjugation pathway for detoxifying the reactive species of the drug (69).

The exact mechanism for acetaminophen-induced hepatic necrosis is unclear, but biotransformation of the drug to a toxic, reactive metabolite has been documented (76). Potter et al. (82) reported that in hamsters acetaminophen may undergo N-hydroxylation by the cytochrome P-450 dependent mixed function oxidase system. It has been suggested that this N-hydroxy derivative of acetaminophen undergoes dehydration spontaneously to form acetamidoquinone (N-acetyl-p-benzoquinoneimine), the metabolite implicated in the development of hepatic necrosis (83). The mechanism proposed by Corcoran et al. (84) and presented in Figure 7 demonstrates the formation of the reactive metabolite N-acetyl-p-benzoquinoneimine. This reactive compound binds covalently with cellular macromolecules, producing cell necrosis (76, 85). Detoxifying the reactive metabolite by glutathione conjugation is believed to prevent cell injury (69).

Even though the exact reactive metabolite of acetaminophen has not been identified, the histological changes occurring in acetaminophen-induced liver necrosis have been examined (71). Walker et al. (70) studied electronmicrographs of the centrilobular regions of the livers of male white mice in order to assess acetaminophen damage. Within two hours, changes were apparent in the centrilobular regions in all animals treated with acetaminophen (500 mg/kg by gavage). Within three hours, there were gross changes in subcellular structures, with both rough and smooth endoplasmic reticuli becoming foamy in appearance and mitochondria appearing swollen. Congestion of the centrilobular regions of the livers was apparent.

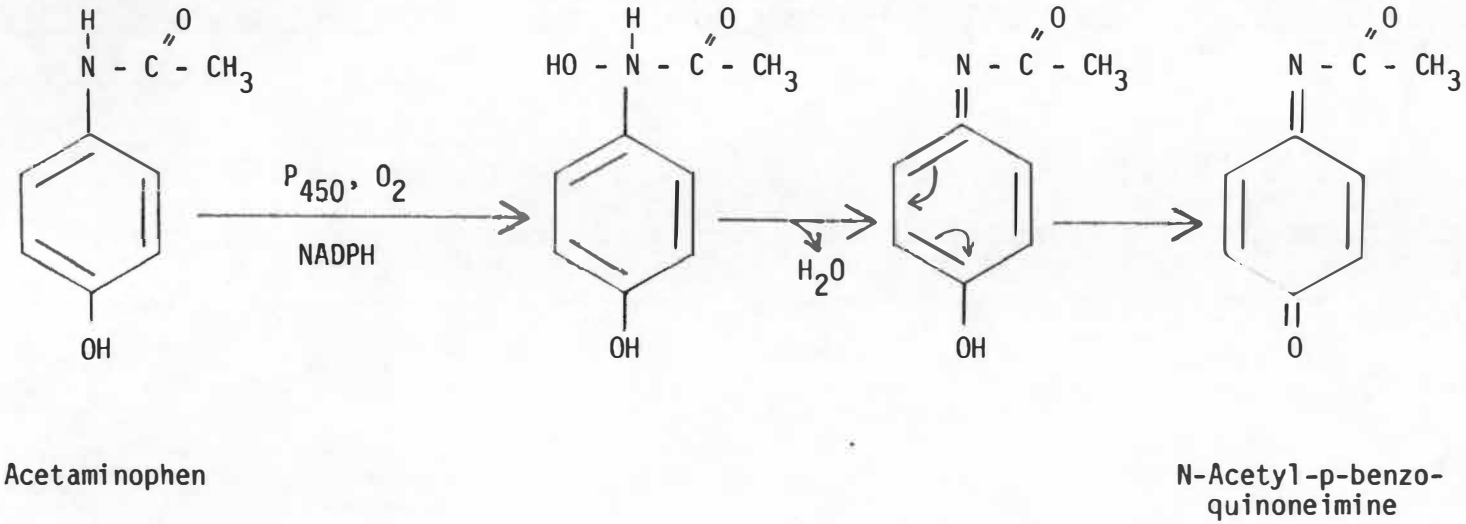


Figure 7. Formation of the reactive metabolite N-acetyl-p-benzoquinoneimine (77).

Treatment of Acetaminophen Overdose

As stated previously, the mechanism of cell damage in acetaminophen overdose is believed to involve the formation of a reactive metabolite (84). It is thought that this reactive species is primarily conjugated with reduced glutathione (GSH) and thereby detoxified. Depletion of GSH results in the covalent binding of the reactive compound to cell macromolecules, thus injuring the cell (81, 82). Depletion of hepatic glutathione following acetaminophen administration has been widely documented (70, 74, 82) and is dose-dependent (71, 86).

Since the discovery of the correlation between GSH availability and the extent of acetaminophen-induced hepatic necrosis, efforts have been made to identify agents that prevent or retard GSH depletion by acetaminophen. Vina et al. (74) reported that in rats the injection of methionine (0.1 g/kg) up to four hours after acetaminophen administration maintained hepatic GSH near physiological levels (73 - 83% of normal). McLean and Day (87) reported that the oral administration of methionine with paracetamol completely prevented hepatic injury in male Wistar rats. There is evidence that methionine acts only indirectly in protecting against hepatic cell damage in mice by serving as a substrate for GSH biosynthesis and sulfate formation. Buckpitt et al. (88) showed that methionine neither reduced covalent binding of the reactive metabolite nor formed acetaminophen adducts.

The effects of cysteine, N-acetylcysteine, and cysteamine on GSH availability and acetaminophen metabolism have also been studied. Mitchell et al. (71) showed that the rate of depletion of liver GSH

by acetaminophen could be significantly reduced by pretreatment of mice with cysteine. All three agents have been shown to reduce the covalent binding of the reactive acetaminophen metabolite in mouse liver (88).

Reports on the mechanism of action of N-acetylcysteine are conflicting. Vina et al. (74) showed that the administration of N-acetylcysteine (1.0 g/kg) to rats did not prevent acetaminophen-induced depletion of hepatic glutathione. The explanation offered for this is that N-acetylcysteine is deacetylated in the liver with the resulting cysteine moiety being oxidized to cystine, a compound not readily used for glutathione biosynthesis according to Thor et al. (89). It has been suggested that N-acetylcysteine does not enhance the recovery of GSH in the livers of rats intoxicated with acetaminophen (74). Galinsky and Levy (72) showed that the oral administration of N-acetylcysteine to adult male rats significantly increased the urinary excretion of acetaminophen sulfate and decreased the excretion of free acetaminophen and the glucuronide conjugate. These researchers concluded that N-acetylcysteine enhanced acetaminophen sulfate formation, thus providing protection against acetaminophen intoxication. It has been suggested that N-acetylcysteine and glutathione both function in preventing hepatic necrosis in acetaminophen overdose by decreasing the covalent binding of the reactive metabolite (88). In spite of the conflict over the mechanism of action of N-acetylcysteine, this agent is considered very efficacious in the treatment of acetaminophen overdose (90).

S-adenosyl-methionine (SAM) has also been investigated as an adjuvant agent in the treatment of acetaminophen toxicity. Stramentinoli et al. (91) reported that the administration of SAM to mice injected with acetaminophen (710 mg/kg) significantly reduced the mortality rate and protected against hepatotoxicity. The mechanism of action for this compound is unknown.

The role of inorganic sulfate in reducing acetaminophen induced hepatotoxicity was studied by Slatterly and Levy (75). These researchers showed that the intraperitoneal injection of sodium sulfate (300 - 800 mg/kg) significantly reduced the incidence of acetaminophen toxicity in male Swiss mice. Moldeus et al. (73) examined acetaminophen conjugation in isolated rat hepatocytes. Their data indicated that the rate of sulfate conjugation was directly correlated with the concentration of sulfate (as magnesium sulfate) in the incubation medium. Addition of either cysteine (1 and 5 mM) or methionine (1 and 5 mM) to the incubation medium did not increase the rate of sulfation. The researchers concluded that inorganic sulfate is required for PAPS formation and hence for acetaminophen sulfate conjugation. These results are in agreement with those reported by Galinsky et al. (92) who studied acetaminophen metabolism in male Sprague-Dawley rats. These researchers reported that the intravenous administration of sodium sulfate to rats enhanced the formation of acetaminophen sulfate and reduced the incidence of acetaminophen toxicity.

CHAPTER III

EXPERIMENTAL PROCEDURE

General Plan

The purpose of this study was to examine the effect of dietary sulfur on the metabolism and urinary excretion of acetaminophen in adult male rats. The basic experimental plan employed a balanced design as discussed previously (see Part I, pages 13-15 and Figure 2, page 14). However, an additional experimental group (Diet 10) was included in this study: one group of rats was fed the 0.027% inorganic sulfate diet with cysteine supplementation (Diet 5) but did not receive an injection of acetaminophen. The inclusion of this group in the experimental plan allowed the researcher to determine whether the vehicle (saline) used in administering the drug produced an effect on the variables being measured.

Male Sprague-Dawley rats (280 - 430 grams) were randomly selected from the rat colony of The University of Tennessee Department of Nutrition and Food Sciences. Rats were randomly assigned to a dietary group and were housed individually in stainless steel cages with wire-mesh bottoms. Distilled water was offered freely. Diets were fed ad libitum for a period of at least 21 days.

Twenty-four hours prior to sacrifice, rats fed Diets 1 - 9 received an intraperitoneal (i.p.) injection of acetaminophen (300 mg/kg) in saline solution in a volume equal to 3% of body weight. This subhepatotoxic dose was the same as that employed by Bolanowska and Gessner (93). Rats fed Diet 10 received an i.p. injection of

saline in a volume equal to 3% of body weight. Following the injection, rats were placed in individual metabolic cages and given free access to the appropriate diet and distilled water.

During the 24-hour period in which rats were housed in the metabolic cages, urine was collected in vials containing 1 ml of 0.1 N HCl as a preservative (94). On the day of sacrifice, urine samples were frozen and stored at -20° until analysis. A modification of the method of Green and Fischer (95) was used to quantitate the primary acetaminophen conjugates in urine by high-performance liquid chromatography (HPLC). Twenty-four hours after injection, rats were killed by decapitation. The livers were removed and divided into two parts: a small section representing the left lobe of the liver (about 2-4 grams) was removed, homogenized in 2% sulfosalicylic acid, and stored in a refrigerator at 4° until the analysis of tissue glutathione concentration could be made; the remaining section was removed, homogenized in 10 mM TRIS buffer, pH 8.0, and centrifuged at 10,000 x g for 1 hour to prepare the supernatant fraction. One ml of the supernatant was used for determining glutathione S-transferase activity according to the method of Habig et al. (50). The remaining supernatant was centrifuged at 105,000 x g for 1 hour. Following this centrifugation, the resulting supernatant was retained for the sulfotransferase assay as outlined by Bolanowska and Gessner (93). The microsomes obtained from this centrifugation were washed in 0.154 M KCl and re-centrifuged at 105,000 x g for 1 hour. The resulting microsomal fraction was suspended in 0.154 M KCl. A sample of this microsomal preparation was used in determining

UDP-glucuronyltransferase activity as described by Bolanowska and Gessner (77).

Description of Diets

The semi-purified diets provided inorganic sulfate at the levels of 0.0072%, 0.027%, and 0.427%. At each level of inorganic sulfate, there was either no organic sulfur supplementation or supplementation with either cysteine or methionine.

The composition of the diets is listed in Tables 1 and 2, pages 17 and 18. Rats were fed the basal diet shown in Table 1 plus one of the diet formulations listed in Table 2. Diets were fed ad libitum for a period of at least 21 days.

Measurement of Glutathione S-Transferase Activity

Twenty-four hours after the injection of either acetaminophen or saline, rats were stunned by a blow to the head and quickly decapitated. The livers were removed and divided into two sections. The small left lobe of the liver (2-4 grams) was removed and retained for the determination of tissue glutathione concentration (see pages 61-65). The remaining large section of the liver was placed in a tared beaker chilled on ice containing about 50 ml of 10 mM TRIS buffer, pH 8.0, and weighed. This liver sample was homogenized with a motor-driven Teflon pestle, diluted with the TRIS buffer to make a 10% homogenate, and centrifuged (Model A, Lourdes Instrument Corp., Brooklyn, NY) at 4^{o} for 1 hour at 10,000 x g.

Following centrifugation, floating lipid was removed and the supernatant decanted into chilled beakers. One ml of the supernatant

was removed for use in the glutathione S-transferase (GSH S-T) assay. The remaining supernatant was retained for use in the UDP-glucuronyltransferase and sulfotransferase assays described below.

The method of Habig et al. (50) was used in determining GSH S-T activity in rat liver. The method was described in detail in Part I, pages 19-20.

Measurement of Sulfotransferase Activity

The supernatant obtained from the initial centrifugation was centrifuged (Model L5-50, Beckman Instruments, Inc., Palo Alto, CA 94304) at 105,000 x g for 1 hour at 4°. The microsomal fraction obtained from this centrifugation was washed in 0.154 M KCl and re-centrifuged in order to prepare the microsomes for use in determining UDP-glucuronyltransferase activity (described below). The supernatant obtained from this centrifugation was removed to chilled beakers and retained for the sulfotransferase assay.

A modification of the method of Bolanowska and Gessner (77, 93) was used to determine sulfotransferase activity in rat liver. One-tenth ml of the supernatant was incubated with 0.9 ml of a reaction mixture containing 2 mM ^{3}H-acetaminophen (2.0 μCi/ml incubation), 0.25 mM PAPS, 5 mM $MgCl_2 \cdot H_2O$, 30 mM nicotinamide, and 5 mM ATP in a 0.08 M potassium phosphate buffer, pH 7.4. The incubations were carried out in a shaker water bath set at 37° for 30 minutes. At the end of this time period, samples were removed from the water bath and placed in an acetone-dry ice bath to stop the reaction by flash freezing. The samples were stored in the freezer at -20° until the analysis could be completed.

To complete the analysis, samples were removed from the freezer, thawed, and saturated with NaCl. Samples were extracted once with 10 ml and twice with 5 ml diethyl ether. With each extraction, the aqueous layer was retained. Following the extraction process, the samples were shaken for 10 minutes. A 0.5 ml sample of the aqueous layer was transferred to a scintillation vial, mixed with a scintillation cocktail containing 6 ml methylcellusol (ethylene glycol monoethylether) and 10 ml PPO (2,5-diphenyloxazole) in toluene (12 g PPO/l toluene), and counted in a liquid-scintillation counter (Model LS 100C, Beckman Instruments, Inc., Fullerton, CA 92634). The channels-ratio method was used to correct for quenching (96).

Measurement of UDP-Glucuronyltransferase Activity

The microsomal fraction obtained from the first ultra-centrifugation was washed in 0.154 M KCl and re-centrifuged for 1 hour at 105,000 x g at 4° . The microsomes obtained from this final centrifugation were suspended in 0.154 M KCl in a volume equivalent to that of the original homogenate. The preparation of this microsomal fraction was described by Gessner (97).

To prepare the incubation medium as described by Bolanowska and Gessner (77), 0.1 ml of the final microsomal preparation was incubated with 0.9 ml of a reaction mixture containing 10 mM ^{3}H-acetaminophen (2.0 μCi/ml incubation), 6 mM UDP-glucuronic acid, and 2 mM UDP-N-acetylglucosamine in a 0.15 M potassium phosphate buffer, pH 7.4. The incubations were carried out in a shaker water bath set at 37° for 15 minutes. At the end of this time period, samples were

removed from the water bath and placed in an acetone-dry ice bath to stop the reaction by flash freezing. The samples were stored in the freezer at -20° until further analysis. Completion of the analysis was the same as that described previously for the sulfotransferase assay.

Determination of Tissue Glutathione Concentration

Tissue glutathione concentration was determined by a modification (98) of the method of Patterson and Lazarow (99). This method is based on the principle that the glyoxalase enzyme, which converts methyl glyoxal to lactic acid, requires reduced glutathione (GSH) as a cofactor. The lactic acid produced in this reaction releases CO_2 from added sodium bicarbonate. Liberated CO_2 is measured manometrically by a Warburg respirometer and is related to GSH concentration (100). Construction of a glutathione standard curve allows the determination of tissue GSH concentration. The reaction catalyzed by the glyoxalase enzyme is shown in Figure 8.

The method of Patterson and Lazarow (99) was followed in preparing the glyoxalase enzyme from baker's yeast. Twelve cakes (about 200 grams) of fresh pressed baker's yeast were crumbled, weighed, and stirred for 10 minutes in 720 ml of cold (4°) acetone. The acetone was removed by suction filtration and the procedure repeated by extracting the residue with 240 ml of cold (4°) acetone. The residue obtained from this extraction was suspended in 288 ml of cold (4°) distilled water and stirred for 10 minutes with a motor-driven stirrer. This process removed glutathione from the

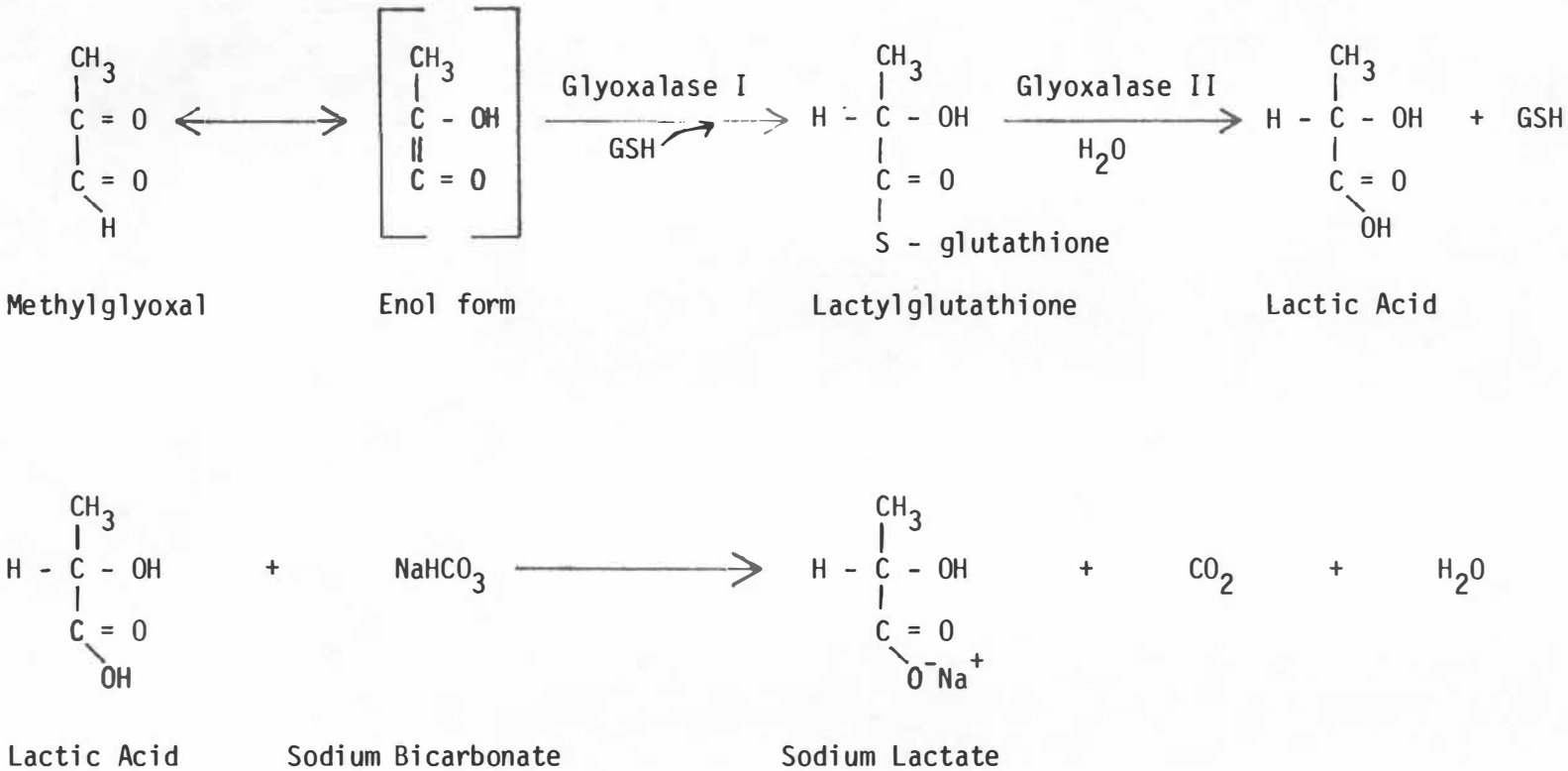


Figure 8. The glyoxalase reaction and production of CO_2 (100).

glyoxalase-containing yeast preparation. The suspension was centrifuged at 4° for 15 minutes at 4,100 x g. Following centrifugation, the water was decanted and the process to remove glutathione from the yeast preparation was repeated 5 times.

After the final centrifugation and removal of water, the residue was suspended in 240 ml of cold (4°) acetone. It was then mixed in a blender on a low setting for 15 seconds. The acetone was removed by suction filtration and the process repeated twice. Following the final removal of acetone by suction filtration, the residue was washed immediately with cold (4°) ether (144 ml). The ether was applied by pouring it over the residue remaining in the suction filter. Finally, the ether-washed residue was placed on bond paper and worked rapidly by hand until dry. The glyoxalase preparation was then quickly weighed, placed in a tinted bottle, and stored at -20°.

In order to determine the tissue concentration of GSH by the glyoxalase method, the Warburg apparatus (American Instrument Co., Inc., Silver Springs, MD) was used. While the water bath of the Warburg apparatus was heating to 25°, one-side-arm flasks without center wells were chilled in an ice bath. A 0.5 ml sample of the glutathione standard or tissue homogenate was pipetted into the side arm. A 1.5 ml sample of the glyoxalase reaction mixture was pipetted into the flask's main compartment. The glyoxalase reaction mixture contained 4.0 g yeast glyoxalase, 11.9 ml 0.2 M $NaHCO_3$, 0.6 ml 40% methylglyoxal, 0.2 ml 2-mercaptoethanol, and 15.0 ml distilled H_2O. One flask containing 2.0 ml distilled water served as the thermobarometer. Duplicates of all samples and standards were run.

When the water bath of the Warburg apparatus reached 25°, the flasks were connected to the manometers and placed in the water bath. While the flasks were allowed to equilibrate for 10 minutes with the system open, the Brodie's solution in the manometers was adjusted to read 150 mm on the right hand scale. The left hand scale served as the zero reading. The contents of the flasks were mixed and allowed to react for 4 minutes at which time the system was closed. Readings from the manometers were taken every 5 minutes for 15 minutes.

The values for tissue glutathione concentration were expressed as μg GSH/sample and mg GSH/g liver tissue (wet weight). The concentration (μg) of GSH in each sample was calculated by first determining the μl CO_2 produced according to the following equation:

$$(K_{flask} + K_{manometer}) \times C = \mu l\ CO_2 \text{ produced}$$

where K_{flask} = a constant for an unwelled flask at 25° C and containing 2 ml fluid; $K_{manometer}$ = a constant for the individual manometer; and C = average change in the reading taken from the manometer. The constants for the manometers and flasks had been previously determined by the method of Umbreit et al. (101). To the value obtained for the μl CO_2 produced/sample, the value representing the change in the thermobarometer was either added or subtracted depending on the pressure change occurring on the day the sample readings were made. The concentration (μg) of GSH/sample was then determined by dividing the adjusted value for μl CO_2 produced by the reaction time (15 min.) to give μl CO_2 produced/minute. This value was then divided by the μl CO_2 produced/mg GSH/min. for the GSH

standard (50 mg GSH/0.5 ml reaction volume) to give μg GSH/sample. The concentration of GSH expressed as mg GSH/g liver tissue (wet weight) was then determined by relating the μg GSH/sample (0.5 ml) to the original homogenate volume.

Determination of Tissue Protein

The method of Lowry et al. (55) was used to determine tissue protein concentration using bovine serum albumin as a standard (200 μg/ml 0.5 N NaOH). The method was described in detail in Part I, pages 19-20.

Measurement of Urinary Acetaminophen Metabolites

A modification of the method of Green and Fischer (95) was used to quantitate the primary acetaminophen conjugates and free acetaminophen in rat urine by high-performance liquid chromatography (HPLC). Frozen urine samples were thawed and diluted 10-fold with HPLC-grade H_2O. One hundred μl of each diluted urine sample was added to a test tube containing 300 μl HPLC-grade H_2O and 100 μl of the internal standard (0.08 M o-toluic acid). As shown in Table 11, several compounds were tested as possible internal standards. O-toluic acid was selected because it was readily soluble in methanol, displayed a relatively good retention time, and produced a peak of acceptable symmetry.

To a final sample volume of 500 μl, 1 ml of absolute ethanol was added. The solution was mixed with a Vortex-Genie for 2 minutes and centrifuged at 1800 RPM for 20 minutes. Following centrifugation, the ethanol was decanted and evaporated to dryness. The resulting residue

TABLE 11

Compounds Tested for Use as an Internal Standard

Compounds Tested[1]	Retention Time	Comments
o-methoxy-benzoic acid	>50 min.	Elutes too late.
m-methoxy-benzoic acid	>50 min.	Elutes too late.
o-toluic acid	30 min.	Possible I. S.
m-toluic acid	>50 min.	Elutes too late.
p-toluic acid	>40 min.	Elutes too late.
o-chloro-benzoic acid	>50 min.	Elutes too late.
p-chloro-benzoic acid	>50 min.	Elutes too late.
p-hydroxy-benzoic acid	12 min.	Elutes too soon.
benzoic acid	35 min.	Interferes with APAP-MA[2] peak.
p-nitro-benzoic acid	35 min.	Interferes with APAP-MA peak.
p-amino-benzoic acid	12 min.	Elutes too soon.

[1]Test conditions were as follows:
Sensitivity = .16
Flow Rate = 1 ml/min.
Solvent = 1% methanol in a buffer of 0.01 M sodium nitrate containing 0.5% acetic acid, pH 2.9.
Chart Speed = 1mm/min.

[2]APAP-MA = Acetaminophen mercapturic acid.

was dissolved in 100 µl HPLC-grade methanol and stored in the freezer (-20°) until the analysis by HPLC.

Prior to sample injection, the mobile phase solvents were degassed for 10 minutes. Two solvent systems were used in the analysis: solvent A (a buffer of 0.5% acetic acid in 0.01 M sodium nitrate, pH 2.9, containing 1% methanol) and solvent B (100% methanol). A gradient approach was employed because it produced good resolution of the compounds of interest while simultaneously promoting the elution of the internal standard within a reasonable time period. The gradient was constructed so that solvent A was pumped through the column at a flow rate of 0.8 ml/min. for 15 minutes following sample injection. After 15 minutes, the flow rate was reduced to 0.6 ml/min. and the level of solvent B was programmed to reach 15% in 15 minutes. When the internal standard had eluted from the column, the flow rate was increased to 0.8 ml/min., the level of solvent B was reduced to 0%, and the system was allowed to equilibrate for 20 minutes prior to the next injection.

A sample volume of 8 µl was injected onto a reverse phase phenyl column (Alltech Associates) with the following specifications:

Guard Column

Size: 5 cm x 4.6 mm I.D.

Packing: Nucleosil Phenyl

Particle Size: 7

Connector

Size: 1/16"

Column

Size: 30 cm x 4.6 mm I.D.

Packing: Nucleosil Phenyl

Particle Size: 7

A Beckman Gradient-Liquid Chromatograph (Model 334) with a system controller (Model 421) was employed. The UV spectrophotometer (Model 153) was equipped with a 254 nm filter. The amount of each metabolite in the urine samples was determined by constructing standard curves using known concentrations of each conjugate and free acetaminophen.[1] The standard curves were made by calculating the ratio of the area of the compound of interest peak to the area of the internal standard peak from the chromatograms. After these values were plotted, the method of least squares was used to fit a line to these points. The SAS program outlined in Appendix D was used to generate equations that described these lines. Given the ratio of compound of interest to internal standard, the concentration of compound of interest was determined by using the appropriate equation. A typical chromatogram is shown in Figure 9.

Statistical Analyses

The effect of acetaminophen on Variable X was evaluated by Student's t-test (56). (Variable X represents any of several variables being measured--e.g., glutathione S-transferase activity, sulfotransferase activity, tissue concentration of reduced

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[1]The acetaminophen sulfate and glucuronide conjugates were a gift from McNeil Consumer Products Company, Fort Washington, PA. The acetaminophen mercapturic acid conjugate was a gift from Winthrop Laboratories, Fawdon, Newcastle Upon Tyne.

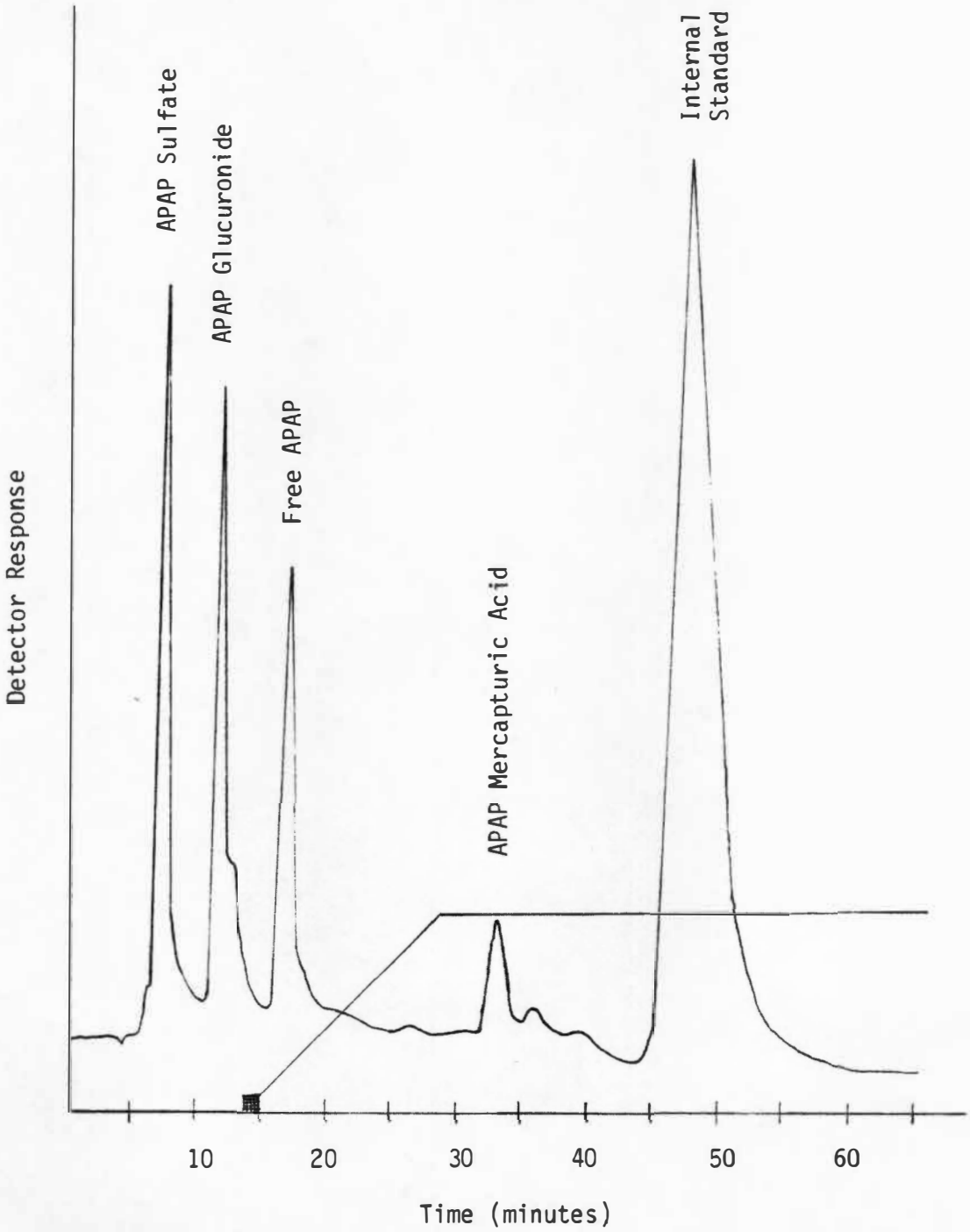

Figure 9. A representative chromatogram demonstrating the major acetaminophen metabolites in rat urine following separation by high-performance liquid chromatography.

glutathione, etc.) The effect of dietary sulfur on Variable X in rats receiving acetaminophen was assessed by the method of planned comparisons (56) as outlined in Part I, pages 20-23. The General Linear Models (GLM) Procedure of the Statistical Analysis System (SAS) package was used to evaluate the data (57).

As in Part I, four central questions were asked of the data. These questions were formulated into contrast statements. The questions followed by the contrast statements are as follows:

1. Does Variable X differ in rats fed diets containing 0.0072% versus 0.027% inorganic sulfate and injected with acetaminophen (Contrast '.0072 vs .027')?
2. Does Variable X differ in rats fed diets containing 0.027% versus 0.427% inorganic sulfate and injected with acetaminophen (Contrast '.027 vs .427')?
3. In rats receiving acetaminophen, does supplementation with organic sulfur affect Variable X (Contrast 'Supple vs No Supple')?
4. In rats receiving acetaminophen, does the nature of the supplementation (cysteine versus methionine) affect Variable X (Contrast 'Cys vs Meth')?

Independence among sample observations was obtained by randomizing both the order in which rats were assigned to dietary groups and the order in which rats were selected for sacrifice on a given day. An alpha level of 0.05 was chosen in establishing statistical significance.

CHAPTER IV

RESULTS

The purpose of this investigation was to determine the effect of dietary sulfur on the hepatic metabolism and urinary excretion of acetaminophen in adult male rats. Rats were fed diets containing 0.0072%, 0.027%, or 0.427% inorganic sulfate with either no organic sulfur supplementation or supplementation with cysteine or methionine. Rats fed Diets 1 - 9 received an intraperitoneal (i.p.) injection of acetaminophen in saline; rats fed Diet 10 received an i.p. injection of saline alone.

In the liver, the activities of the glutathione S-transferases (GSH S-T), sulfotransferase (ST), and UDP-glucuronyltransferase (UDP-GT) were determined. The hepatic concentration of reduced glutathione (GSH) was also measured. In the urine, free acetaminophen, acetaminophen glucuronide, acetaminophen sulfate, and acetaminophen mercapturic acid were quantitated by high-performance liquid chromatography (HPLC).

The study was designed to answer the following questions where Variable X = any variable being measured:

1. Does Variable X differ in rats fed diets containing 0.0072% versus 0.027% inorganic sulfate and injected with acetaminophen (Contrast '.0072 vs .027')?
2. Does Variable X differ in rats fed diets containing 0.027% versus 0.427% inorganic sulfate and injected with acetaminophen (Contrast '.027 vs .427')?

3. In rats receiving acetaminophen, does supplementation with organic sulfur affect Variable X (Contrast 'Supple vs No Supple')?
4. In rats receiving acetaminophen, does the nature of the supplementation (cysteine versus methionine) affect Variable X (Contrast 'Cys vs Meth')?

Effect of Acetaminophen Administration on Hepatic GSH Content and Enzyme Activities

As shown in Table 12, the i.p. administration of acetaminophen to adult male rats significantly increased hepatic GSH concentration when the data were analyzed by Student's t-test. This was true whether the data were expressed as μg GSH/sample or mg GSH/g liver tissue (wet weight).

Tables 13, 14, and 15 show that the i.p. administration of acetaminophen to adult male rats did not affect the hepatic activities of GSH S-T, ST, and UDP-GT respectively. Student's t-test was used to compare means (56).

Effect of Dietary Sulfur on Hepatic Glutathione S-Transferase Activity in Rats Receiving Acetaminophen

The data for hepatic GSH S-T activity in rats receiving acetaminophen are shown in Table 16. Table 17 presents the analysis of variance summary for these data.

As seen in Table 17, dietary inorganic sulfate appeared to have no effect on GSH S-T activity when the data were analyzed by the method of planned comparisons. However, supplementation of diets with organic sulfur significantly reduced GSH S-T activity in the livers of

TABLE 12

The Effect of Acetaminophen Administration on the Hepatic Concentration of Reduced Glutathione (GSH) in Rats

Diet	GSH Concentration	Degrees of Freedom	T-Value[1]	P-Value
	μg GSH/sample			
5 (with APAP[2])	147 ± 8[3](6)[4]	9.0	3.6160	0.0056
10 (without APAP)	95 ± 13 (5)			
	mg GSH/g liver (wet weight)			
5 (with APAP)	2.93 ± .15 (6)	9.0	3.4581	0.0072
10 (without APAP)	1.94 ± .26 (5)			

[1]Means were compared using Student's t-test (56).

[2]APAP = Acetaminophen.

[3]Values represent mean ± SEM.

[4]Number in parentheses indicates the number of experimental animals used.

TABLE 13

The Effect of Acetaminophen Administration on Glutathione S-Transferase Activity in Rat Liver

Diet	GSH S-T Activity[1]	Degrees of Freedom	T-Value[2]	P-Value
5 (With APAP[3])	.323 ± .09[4](6)[5]			
		9.0	0.5068	0.6245
10 (Without APAP)	.266 ± .06 (5)			

[1]GSH S-T Activity = μmoles 1-chloro-2,4-dinitrobenzene conjugated/mg protein/minute.

[2]Means were compared using Student's t-test (56).

[3]APAP = Acetaminophen.

[4]Values represent mean ± SEM.

[5]Number in parentheses indicates the number of experimental animals used.

TABLE 14

The Effect of Acetaminophen Administration on Sulfotransferase Activity in Rat Liver

Diet	S-T Activity[1]	Degrees of Freedom	T-Value[2]	P-Value
5 (With APAP[3])	0.029 ± 0.005[4](6)[5]			
		9.0	-0.3230	0.7541
10 (Without APAP)	0.032 ± 0.007 (5)			

[1]S-T Activity = nmoles acetaminophen sulfate formed/mg protein/minute.

[2]Means were compared using Student's t-test (56).

[3]APAP = Acetaminophen.

[4]Values represent mean ± SEM.

[5]Number in parentheses indicates the number of experimental animals used.

TABLE 15

The Effect of Acetaminophen Administration on UDP-Glucuronyltransferase Activity in Rat Liver

Diet	UDP-GT Activity[1]	Degrees of Freedom	T-Value[2]	P-Value
5 (With APAP[3])	54 ± 12[4](6)[5]			
		9.0	0.4543	0.6604
10 (Without APAP)	49 ± 6 (5)			

[1]UDP G-T Activity = nmoles acetaminophen glucuronide formed/mg protein/minute.

[2]Means were analyzed by Student's t-test (56).

[3]APAP = Acetaminophen.

[4]Values represent mean ± SEM.

[5]Number in parentheses indicates the number of experimental animals used.

TABLE 16

The Effect of Dietary Sulfur on Hepatic Glutathione S-Transferase Activity Expressed as μmoles CDNB[1] Conjugated/mg Protein/Minute in Rats Receiving Acetaminophen

Organic Sulfur	Level of Dietary Inorganic Sulfate			
	0.0072%	0.027%	0.427%	Group Means
	moles CDNB conjugated/mg protein/minute			
None	.467 ± .07[2](5)[3]	.393 ± .06 (6)	.325 ± .07 (5)	.395 ± .04 (16)
Cysteine (0.5 g/100 g diet)	.232 ± .07 (5)	.323 ± .06 (6)	.235 ± .06 (6)	.263 ± .04 (17)
Methionine (0.6 g/100 g diet)	.357 ± .08 (4)	.345 ± .06 (6)	.357 ± .06 (6)	.353 ± .04 (16)
Group Means	.352 ± .04 (14)	.354 ± .04 (18)	.305 ± .04 (17)	

[1]CDNB = 1-Chloro-2,4-dinitrobenzene.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 17

Analysis of Variance Summary for Hepatic Glutathione S-Transferase Activity Expressed as μmoles CDNB[1] Conjugated/mg Protein/Minute in Rats Receiving Acetaminophen

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	0.0252	0.51	0.6026
Supplementation	2	0.1486	3.03	0.0596
Interaction	4	0.0586	0.60	0.6666
Contrasts Between Group Means				
'.0072 vs .027'	1	0.00003	0.00	0.9703
'.027 vs .427'	1	0.02060	0.84	0.3648
'Supple vs No Supple'	1	0.09985	4.26	0.0487
'Cys vs Meth'	1	0.06491	2.65	0.1116

[1]CDNB = 1-Chloro-2,4-dinitrobenzene.

rats receiving acetaminophen. Furthermore, supplementation of diets with cysteine produced a greater reduction in GSH S-T activity than did methionine supplementation. This observed effect in the difference between the effect of cysteine and methionine supplementation on enzyme activity is in keeping with that seen in the Part I data (see Table 7, page 32).

Effect of Dietary Sulfur on Hepatic GSH Content in Rats Receiving Acetaminophen

The data for hepatic GSH content expressed as μg GSH/sample are given in Table 18; Table 19 presents the analysis of variance summary for these data. The data for GSH concentration expressed as mg GSH/g liver tissue (wet weight) are shown in Table 20; the analysis of variance summary for these data is given in Table 21.

An examination of Tables 19 and 21 reveals that dietary inorganic sulfate appeared to have no effect on hepatic GSH levels when the data were analyzed by the method of planned comparisons. However, GSH levels were significantly greater in the livers of rats fed diets supplemented with organic sulfur than in rats fed diets deficient in organic sulfur. There appeared to be no difference in the effect of cysteine and methionine supplementation on hepatic GSH levels.

In addition, an examination of the data shown in Tables 18 and 20 reveals that in the livers of rats fed diets supplemented with cysteine, GSH levels were highest at the 0.027% level of dietary inorganic sulfate. When diets were supplemented with methionine, hepatic GSH levels were lowest at the 0.027% inorganic sulfate level.

TABLE 18

The Effect of Dietary Sulfur on Hepatic Glutathione (GSH) Concentration Expressed as μg GSH/Sample in Rats Receiving Acetaminophen

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027%	0.427%	
	μg GSH/sample			
None	82 ± 10[1](5)[2]	94 ± 9 (6)	97 ± 10 (5)	91 ± 6 (16)
Cysteine (0.5 g/100 g diet)	115 ± 10 (5)	147 ± 9 (6)	74 ± 9 (6)	112 ± 5 (17)
Methionine (0.6 g/100 g diet)	106 ± 11 (4)	84 ± 9 (6)	133 ± 9 (5)	108 ± 6 (16)
Group Means	101 ± 6 (14)	108 ± 5 (18)	102 ± 5 (17)	

[1]Values represent mean ± SEM.

[2]Number in parentheses indicates the number of experimental animals used.

TABLE 19

Analysis of Variance Summary for Hepatic Glutathione Concentration Expressed as μg GSH/Sample in Rats Receiving Acetaminophen

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	499.2864	00.51	0.6055
Supplementation	2	4117.7391	04.19	0.0223
Interaction	4	23498.3418	11.96	0.0001
Contrasts Between Group Means				
'.0072 vs .027'	1	369.0087	00.75	0.3913
'.027 vs .427'	1	359.2301	00.73	0.3976
'Supple vs No Supple'	1	3904.3363	07.95	0.0075
'Cys vs Meth'	1	168.8995	00.34	0.5610

TABLE 20

The Effect of Dietary Sulfur on Hepatic Glutathione (GSH) Concentration Expressed as mg GSH/g Liver Tissue (Wet Weight) in Rats Receiving Acetaminophen

	Level of Dietary Inorganic Sulfate			
Organic Sulfur	0.0072%	0.027%	0.427%	Group Means
	mg GSH/g liver (wet weight)			
None	1.62 ± 0.20[1](5)[2]	1.90 ± 0.19 (6)	1.90 ± 0.20 (5)	1.81 ± 0.11 (16)
Cysteine (0.5 g/100 g diet)	2.30 ± 0.20 (5)	2.93 ± 0.19 (6)	1.47 ± 0.19 (6)	2.23 ± 0.11 (17)
Methionine (0.6 g/100 g diet)	2.15 ± 0.23 (4)	1.70 ± 0.19 (6)	2.65 ± 0.19 (6)	2.17 ± 0.12 (16)
Group Means	2.02 ± 0.12 (14)	2.18 ± 0.11 (18)	2.01 ± 0.11 (17)	

[1]Values represent mean ± SEM.

[2]Number in parentheses indicates the number of experimental animals used.

TABLE 21

Analysis of Variance Summary for Hepatic Glutathione Concentration Expressed as mg GSH/g Liver Tissue (Wet Weight) in Rats Receiving Acetaminophen

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	0.3085	0.75	0.4812
Supplementation	2	1.6956	4.10	0.0241
Interaction	4	9.1334	11.03	0.0001
Contrasts Between Group Means				
'.0072 vs .027'	1	0.1867	0.90	0.3480
'.027 vs .427'	1	0.2583	1.25	0.2706
'Supple vs No Supple'	1	1.6462	7.95	0.0074
'Cys vs Meth'	1	0.0358	0.17	0.6796

Effect of Dietary Sulfur on Hepatic Sulfotransferase Activity in Rats Receiving Acetaminophen

The data showing the effect of dietary sulfur on hepatic sulfotransferase activity in rats receiving acetaminophen are presented in Table 22. The analysis of variance summary for these data is given in Table 23 and shows that when the data were analyzed by the method of planned comparisons, sulfotransferase activity did not appear to be affected by either the level of dietary inorganic sulfate or supplementation with organic sulfur.

Effect of Dietary Sulfur on Hepatic UDP-Glucuronyltransferase Activity in Rats Receiving Acetaminophen

The data showing the effect of dietary sulfur on hepatic UDP-GT activity in rats receiving acetaminophen are presented in Table 24. The analysis of variance summary for these data is given in Table 25 and shows that when the data were analyzed by the method of planned comparisons, UDP-GT activity did not appear to be affected by either the level of dietary inorganic sulfate or supplementation with organic sulfur.

Effect of Dietary Sulfur on the Urinary Excretion of Acetaminophen by Adult Male Rats

Table 26 shows the effect of dietary sulfur on the urinary excretion of acetaminophen sulfate by adult male rats. The analysis of variance summary for these data is given in Table 27. When the data were analyzed by the method of planned comparisons as shown in Table 27, the urinary excretion of acetaminophen sulfate did not appear to be affected by either the level of dietary inorganic sulfate or supplementation with organic sulfur.

TABLE 22

The Effect of Dietary Sulfur on Hepatic Sulfotransferase Activity in Rats Receiving Acetaminophen

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027$	0.427%	
	nmoles APAP[1] conjugated/mg protein/minute			
None	.035 ± .007[2](5)[3]	.033 ± .007 (6)	.037 ± .009 (5)	.035 ± .005 (16)
Cysteine (0.5 g/100 g diet)	.036 ± .008 (5)	.029 ± .007 (6)	.037 ± .007 (6)	.034 ± .004 (17)
Methionine (0.6 g/100 g diet)	.032 ± .009 (4)	.041 ± .007 (6)	.037 ± .007 (6)	.037 ± .005 (16)
Group Means	.034 ± .005 (14)	.034 ± .004 (18)	.037 ± .005 (17)	

[1]APAP = Acetaminophen.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 23

Analysis of Variance Summary for Sulfotransferase Data

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	0.00007	0.11	0.8971
Supplementation	2	0.00006	0.09	0.9153
Interaction	4	0.00038	0.29	0.8801
Contrasts Between Group Means				
'.0072 vs .027'	1	0.00005	0.17	0.6858
'.027 vs .427'	1	0.00000	0.01	0.9073
'Supple vs No Supple'	1	0.00000	0.00	0.9965
'Cys vs Meth'	1	0.00006	0.17	0.6813

TABLE 24

The Effect of Dietary Sulfur on Hepatic UDP-Glucuronyltransferase Activity in Rats Receiving Acetaminophen

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027%	0. 427%	
	nmoles APAP[1] conjugated/mg protein/minute			
None	56 ± 10[2](5)[3]	47 ± 9 (6)	50 ± 10 (5)	51 ± 6 (16)
Cysteine (0.5 g/100 g dietO	38 ± 10 (5)	55 ± 9 (6)	50 ± 9 (6)	48 ± 5 (17)
Methionine (0.6 g/100 g diet)	48 ± 11 (4)	60 ± 9 (6)	52 ± 9 (6)	53 ± 6 (16)
Group Means	48 ± 6 (14)	54 ± 5 (18)	50 ± 5 (17)	

[1]APAP = Acetaminophen.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 25

Analysis of Variance Summary for UDP-Glucuronyltransferase Data

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	298.1475	0.30	0.7461
Supplementation	2	248.9279	0.25	0.7828
Interaction	4	1026.5825	0.51	0.7301
Contrasts Between Group Means				
'.0072 vs .027'	1	292.8022	0.58	0.4510
'.027 vs .427'	1	98.0688	0.19	0.6619
'Supple vs No Supple'	1	3.0582	0.01	0.9384
'Cys vs Meth'	1	244.2988	0.48	0.4909

TABLE 26

The Effect of Dietary Sulfur on the Urinary Excretion of Acetaminophen Sulfate by Adult Male Rats

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027%	0.427%	
	mg APAP-SO_4[1] excreted/24 hours			
None	5.7 ± 1.3[2](5)[3]	5.1 ± 1.2 (6)	5.6 ± 1.3 (5)	5.5 ± 0.7 (16)
Cysteine (0.5 g/100 g diet)	8.1 ± 1.3 (5)	5.3 ± 1.2 (6)	5.5 ± 1.2 (6)	6.3 ± 0.7 (17)
Methionine (0.6 g/100 g diet)	5.8 ± 1.4 (4)	5.4 ± 1.2 (6)	6.9 ± 1.2 (6)	6.0 ± 0.7 (16)
Group Means	6.5 ± 0.8 (14)	5.2 ± 0.7 (18)	6.0 ± 0.7 (17)	

[1]APAP-SO_4 = Acetaminophen sulfate.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 27

Analysis of Variance Summary for the Urinary Excretion of Acetaminophen Sulfate by Adult Male Rats

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	13.6276	0.85	0.4368
Supplementation	2	5.4093	0.34	0.7168
Interaction	4	19.9616	0.62	0.6513
Contrasts Between Group Means				
'.0072 vs .027'	1	13.1351	1.63	0.2090
'.027 vs .427'	1	5.2238	0.65	0.4255
'Supple vs No Supple'	1	4.8960	0.61	0.4403
'Cys vs Meth'	1	0.4346	0.05	0.8175

The data showing the effect of dietary sulfur on the urinary excretion of acetaminophen glucuronide by adult male rats are given in Table 28. An examination of the analysis of variance summary for these data (Table 29) reveals an effect of both dietary inorganic sulfate and organic sulfur supplementation on the urinary excretion of acetaminophen glucuronide. Excretion of the glucuronide conjugate was significantly increased in rats fed diets containing 0.0072% inorganic sulfate (Contrast '.0072 vs .027'). In addition, rats fed diets deficient in organic sulfur excreted significantly more acetaminophen glucuronide than rats fed diets supplemented with cysteine and methionine. Also, the type of organic sulfur supplementation had a significant effect on acetaminophen glucuronide excretion. Rats fed diets supplemented with cysteine excreted significantly more of the glucuronide conjugate than did rats fed diets supplemented with methionine.

The data showing the effect of dietary sulfur on the urinary excretion of free acetaminophen are presented in Table 30. The analysis of variance summary for these data is given in Table 31 and shows that the excretion of free acetaminophen was significantly reduced in rats fed diets containing inorganic sulfate at the level of 0.427%. Supplementation of diets with organic sulfur did not appear to affect the urinary excretion of free acetaminophen when the data were analyzed by the method of planned comparisons.

Table 32 shows the effect of dietary sulfur on the urinary excretion of acetaminophen mercapturic acid by adult male rats. The analysis of variance summary for these data are presented in Table 33.

TABLE 28

The Effect of Dietary Sulfur on the Urinary Excretion of Acetaminophen Glucuronide by Adult Male Rats

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027%	0.427%	
	mg APAP-GU[1] excreted/24 hours			
None	6.7 ± 0.7[2](5)[3]	4.5 ± 0.7 (6)	3.9 ± 0.7 (5)	5.0 ± 0.4 (16)
Cysteine (0.5 g/100 g diet)	5.5 ± 0.7 (5)	4.5 ± 0.7 (6)	4.0 ± 0.7 (6)	4.7 ± 0.4 (17)
Methionine (0.6 g/100 g diet)	3.2 ± 0.8 (4)	2.5 ± 0.7 (6)	3.5 ± 0.7 (6)	3.1 ± 0.4 (16)
Group Means	5.1 ± 0.4 (14)	3.8 ± 0.4 (18)	3.8 ± 0.4 (17)	

[1]APAP-GU = Acetaminophen glucuronide.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 29

Analysis of Variance Summary for the Urinary Excretion of Acetaminophen Glucuronide by Adult Male Rats

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	16.8754	3.04	0.0591
Supplementation	2	33.8710	6.10	0.0049
Interaction	4	12.4482	1.12	0.3605
Contrasts Between Group Means				
'.0072 vs .027'	1	13.2148	4.76	0.0351
'.027 vs .427'	1	0.0010	0.00	0.9844
'Supple vs No Supple'	1	14.3382	5.16	0.0285
'Cys vs Meth'	1	20.3899	7.34	0.0099

TABLE 30

The Effect of Dietary Sulfur on the Urinary Excretion of Free Acetaminophen by Adult Male Rats

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027%	0.427%	
	mg free APAP[1] excreted/24 hours			
None	3.6 ± 0.4[2](5)[3]	2.8 ± 0.4 (6)	1.6 ± 0.4 (5)	2.6 ± 0.2 (16)
Cysteine (0.5 g/100 g diet)	3.1 ± 0.4 (5)	3.1 ± 0.4 (6)	2.2 ± 0.4 (6)	2.8 ± 0.2 (17)
Methionine (0.6 g/100 g diet)	2.6 ± 0.4 (4)	2.8 ± 0.4 (6)	2.2 ± 0.4 (6)	2.6 ± 0.2 (16)
Group Means	3.1 ± 0.2 (14)	2.9 ± 0.2 (18)	2.0 ± 0.2 (17)	

[1]APAP = Acetaminophen.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 31

Analysis of Variance Summary for the Urinary Excretion of Free Acetaminophen by Adult Male Rats

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	11.2095	7.13	0.0022
Supplementation	2	0.6098	0.39	0.6810
Interaction	4	3.4013	1.08	0.3785
Contrasts Between Group Means				
'.0072 vs .027'	1	0.2792	0.36	0.5546
'.027 vs .427'	1	7.2654	9.24	0.0042
'Supple vs No Supple'	1	0.0210	0.03	0.8709
'Cys vs Meth'	1	0.5827	0.74	0.3944

TABLE 32

The Effect of Dietary Sulfur on the Urinary Excretion of Acetaminophen Mercapturic Acid by Adult Male Rats

Organic Sulfur	Level of Dietary Inorganic Sulfate			Group Means
	0.0072%	0.027%	0.427%	
	mg APAP-MA[1] excreted/24 hours			
None	1.7 ± 0.1[2](5)[3]	1.4 ± 0.1 (6)	1.0 ± 0.1 (5)	1.4 ± 0.1 (16)
Cysteine (0.5 g/100 g diet)	1.1 ± 0.1 (5)	1.0 ± 0.1 (6)	0.7 ± 0.1 (6)	0.9 ± 0.1 (17)
Methionine (0.6 g/100 g diet)	0.8 ± 0.1 (4)	1.0 ± 0.1 (6)	1.1 ± 0.1 (6)	0.9 ± 0.1 (16)
Group Means	1.2 ± 0.1 (14)	1.1 ± 0.1 (18)	0.9 ± 0.1 (17)	

[1]APAP-MA = Acetaminophen mercapturic acid.

[2]Values represent mean ± SEM.

[3]Number in parentheses indicates the number of experimental animals used.

TABLE 33

Analysis of Variance Summary for the Urinary Excretion of Acetaminophen Mercapturic Acid by Adult Male Rats

Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
Sulfate Level	2	0.5690	3.22	0.0504
Supplementation	2	1.8028	10.21	0.0003
Interaction	4	1.3146	3.72	0.0115
Contrasts Between Group Means				
'.0072 vs .027'	1	0.0937	1.06	0.3090
'.027 vs .427'	1	0.2229	2.52	0.1199
'Supple vs No Supple'	1	1.7765	20.12	0.0001
'Cys vs Meth'	1	0.0163	0.18	0.6696

When the data were analyzed by the method of planned comparisons, dietary inorganic sulfate appeared to have no effect on the excretion of the mercapturic acid conjugate. Supplementation of diets with organic sulfur (cysteine + methionine) significantly reduced the excretion of this metabolite.

As shown in Table 34, the major urinary metabolite of acetaminophen was acetaminophen sulfate (32 - 50% of the total dose excreted). Acetaminophen glucuronide was the next largest drug component (20 - 38% of the total dose excreted), followed by free acetaminophen (13 - 27%) and acetaminophen mercapturic acid (6 - 10%). The ratio of acetaminophen glucuronide to acetaminophen sulfate (AG:AS) excreted varied among the diets. The ratio was greatest in rats fed Diet 1 (0.0072% $SO_4^=$ without organic sulfur supplementation) and lowest in rats fed Diet 6 (0.027% $SO_4^=$ + methionine).

TABLE 34

Percent of Total Dose Excreted as Each Acetaminophen Metabolite

Diet	Level of Dietary Sulfate	DL-Methionine	Cysteine	APAP-SO_4[1]	APAP-GU[2]	APAP-Free[3]	APAP-MA[4]	AG:AS Ratio[5]
	%	- - - - g/100 g diet - - -		- - - - - - - - - - % Excreted - - - - - - - - - -				
1	0.0072	0.0	0.0	32 ± 8[6]	38 ± 7	20 ± 1	10 ± .5	1.35
2	0.0072	0.0	0.53	47 ± 10	29 ± 7	17 ± 4	6 ± 1	0.24
3	0.0072	0.65	0.0	45 ± 10	26 ± 2	22 ± 9	7 ± 1	0.23
4	0.027	0.0	0.0	37 ± 2	33 ± 4	20 ± 3	10 ± 2	0.12
5	0.027	0.0	0.50	36 ± 13	33 ± 9	24 ± 9	7 ± 2	1.03
6	0.027	0.62	0.0	44 ± 9	20 ± 5	27 ± 9	9 ± 4	0.09
7	0.427	0.0	0.0	47 ± 11	32 ± 7	13 ± 3	8 ± 2	0.34
8	0.427	0.0	0.50	41 ± 11	35 ± 10	18 ± 6	6 ± 1	0.41
9	0.427	0.62	0.0	50 ± 5	24 ± 5	17 ± 4	9 ± 2	0.12

[1]APAP-SO_4 = Acetaminophen sulfate.

[2]APAP-GU = Acetaminophen glucuronide.

[3]APAP-Free = Free acetaminophen.

[4]APAP-MA = Acetaminophen mercapturic acid.

[5]AG:AS Ratio = Ratio of acetaminophen glucuronide to acetaminophen sulfate excreted.

[6]Values represent mean ± S. D.

CHAPTER V

DISCUSSION

Acetaminophen is widely accepted as an analgesic and anti-pyretic agent and exhibits few side effects when taken theraputically (85). Normally, acetaminophen is rapidly absorbed and metabolized by the liver; the metabolic end-products are excreted by the kidney (102). In most species, acetaminophen sulfate and acetaminophen glucuronide account for about 75-80% of the total dose excreted. Acetaminophen mercapturic acid usually constitutes 3-4% of the total dose excreted by rats (81).

In large doses, acetaminophen damages the liver in both man and experimental animals (69, 70, 71). The exact mechanism by which the centrilobular regions of the liver are damaged by excessive acetaminophen ingestion remains unclear (85). However, there is evidence that a cytochrome P-450-activated metabolite of acetaminophen binds to cell macromolecules, resulting in cell death (103). The tripeptide glutathione appears to offer some protection against acetaminophen-induced hepatic necrosis (82).

A number of agents have been used in the treatment of acetaminophen toxicity. These include N-acetylcysteine (69), methionine (73), cysteine (73), S-adenosylmethionine (91), and sulfate (73, 75). It has been demonstrated, for example, that the oral administration of N-acetylcysteine (72) and the intraperitoneal injection of sodium sulfate (73) alter the excretion pattern of

acetaminophen conjugates in laboratory animals. With the observation that cytochrome P-450 levels (4) and the glucuronidation of salicylamide vary as a function of the level of dietary inorganic sulfate (11), the researcher posed the following question: Does dietary sulfur alter the hepatic metabolism and urinary excretion of acetaminophen in adult male rats? In an effort to answer this question, the present study was undertaken.

In this investigation, dietary inorganic sulfate appeared to have no effect on the hepatic activities of the glutathione S-transferases (GSH S-T), sulfotransferase (ST), and UDP-glucuronyltransferase (UDP-GT) in rats receiving a sub-hepatotoxic dose of acetaminophen. In addition, the hepatic level of reduced glutathione (GSH) was also unaffected by the level of dietary inorganic sulfate. One possible explanation for these results is that the sample size was too small to detect meaningful differences in variable response given the level of within-group variability and the conservative nature of the statistical test. Also, regarding GSH concentration, there was a significant interaction effect, (Table 19, page 81, and Table 21, page 83), suggesting that the effects of dietary inorganic sulfate and dietary organic sulfur on hepatic GSH levels cannot be totally separated.

In the urine, dietary inorganic sulfate appeared to have no effect on the excretion of acetaminophen sulfate (Table 27, page 90). However, the urinary levels of acetaminophen glucuronide, free acetaminophen, and acetaminophen mercapturic acid were affected by the level of dietary inorganic sulfate when the data were analyzed by the

method of planned comparisons. In general, the excretion of acetaminophen glucuronide (Table 28, page 92), free acetaminophen (Table 30, page 94), and the mercapturic acid conjugate (Table 32, page 96) was greatest in rats fed diets containing inorganic sulfate at the level of 0.0072%. Also, the urinary excretion of free acetaminophen and the mercapturic acid conjugate decreased as the level of dietary inorganic sulfate increased from 0.0072% to 0.427%.

These results are, in part, fairly predictable. With the observation that the rate of sulfation in isolated rat hepatocytes is dependent on the availability of inorganic sulfate for PAPS formation (73), it is not surprising that the excretion of free acetaminophen, and the glucuronide and mercapturic acid conjugates is increased at the lowest (0.0072%) level of dietary inorganic sulfate. This most likely represents a compensatory response to the reduced availability of inorganic sulfate for acetaminophen sulfate formation. However, the observation that the level of dietary inorganic sulfate had no apparent effect on the urinary excretion of acetaminophen sulfate is somewhat unexpected. With the exception of Diet 2 (0.0072% $SO_4^=$ + cysteine) and Diet 9 (0.427% $SO_4^=$ + methionine), the values for acetaminophen sulfate excretion were fairly consistent across all diets (Table 26, page 89). The data indicate that the sulfation pathway is operating at full capacity given the dosage level of acetaminophen. Other researchers have reported that in rats the acetaminophen sulfate conjugating pathway is saturated at drug doses of 300 - 600 mg/kg (80, 81, 93, 97).

In this study, the supplementation of diets with organic sulfur altered the hepatic metabolism and urinary excretion of acetaminophen

in rats. In the liver, GSH S-T activity was significantly reduced in rats fed diets sufficient in organic sulfur (Table 17, page 78). For the most part, this result is in keeping with that reported in Part I (Table 10, page 35). The data indicate that there is a compensatory increase in GSH S-T activity in the livers of rats fed diets deficient in organic sulfur. This increase in the in vitro rate of the enzyme-catalyzed reaction may reflect an in vivo acceleration of protein synthesis, retardation of protein degradation, or enhancement of enzyme activation (62).

In addition, the availability of substrate(s) affects enzyme activity in vivo (62). Since GSH S-T activity had been shown to vary as a function of dietary sulfur (see Part I), it was of interest to measure the availability of the substrate GSH. In this study, liver GSH levels were significantly reduced in rats fed diets deficient in organic sulfur and receiving acetaminophen (Table 21, page 83). The absolute values seen for this group (Table 20, page 82) were only slightly lower than the value reported for rats receiving an injection of saline alone (Diet 10, Table 12, page 73). In addition, the hepatic levels of GSH seen in rats fed diets deficient in organic sulfur are in agreement with GSH values of 1.39 mg/g liver - 2.65 mg/g liver reported previously in the literature (74, 104, 105, 106, 107, 108). The data indicate that GSH biosynthesis is increased in the livers of rats fed diets supplemented with organic sulfur and injected with acetaminophen. On first inspection, this observation does not appear to agree with published reports that hepatic GSH depletion by

acetaminophen is dose- and time-dependent (70, 71, 82, 86). However, the results of a study by Vina et al. (74) indicate that while acetaminophen depletion of GSH in rat liver is maximal at 2 - 10 hours, hepatic GSH values return to normal within 24 hours. Potter et al. (82) have reported that GSH levels are 161% of normal at 24 hours in hamsters following acetaminophen administration (300 mg/kg, i.p.). Thus, the values for GSH concentration presented in this study may reflect an enhanced biosynthesis of GSH in rat liver during a recovery phase following acetaminophen administration.

In the urine, the excretion of acetaminophen glucuronide and acetaminophen mercapturic acid was increased in rats fed diets deficient in organic sulfur. If, as was postulated previously, hepatic GSH S-T activity was increased in rats fed diets deficient in organic sulfur and reflected increased acetaminophen binding to the enzyme in the absence of a physiological level of GSH, the increased urinary excretion of the mercapturic acid conjugate in rats fed these diets may represent enhanced formation of the glutathione conjugate following the recovery of hepatic GSH levels after 24 hours. The increased urinary excretion of acetaminophen glucuronide in rats fed diets deficient in organic sulfur is more difficult to explain. Moldeus et al. (73) reported that in isolated hepatocytes, the rate of glucuronidation of acetaminophen was dependent on the availability of UDP-glucuronic acid (UDP-GA). In this study, the increase seen in the urinary excretion of acetaminophen glucuronide in rats fed diets deficient in organic sulfur may reflect enhanced synthesis of the substrate UDP-GA.

Since methionine (73, 74) and cysteine (73, 87) have both been used in the treatment of acetaminophen toxicity, it was of interest to assess the effect of the type of dietary organic sulfur supplementation on the metabolism and excretion of this drug. Data collected previously in this laboratory (11) and the data presented in Part I indicate that dietary cysteine and methionine do not exert equivalent metabolic effects in the rat. The present study provides additional evidence in support of this observation. Specifically, the urinary excretion of acetaminophen glucuronide (Table 28, page 92) was significantly greater in rats fed diets supplemented with cysteine than in rat fed diets supplemented with methionine. Although hepatic UDP-GT activity did not exhibit a similar response to the type of dietary organic sulfur supplementation (Table 25, page 88) in this study, other work in this laboratory has shown an increase in UDP-GT activity in rats fed diets supplemented with cysteine (11).

In general, the excretion pattern of drug metabolites seen in this study is in agreement with values reported in the literature. Jollow et al. (81) reported that in rats receiving an i.p. injection of acetaminophen (50 mg), acetaminophen sulfate accounted for about 48% of the total dose excreted. Acetaminophen glucuronide accounted for 35% of the total dose excreted, followed by free acetaminophen (12%) and the mercapturic acid conjugate (4%). In this investigation, the excretion of free acetaminophen and acetaminophen mercapturic acid were slightly elevated when compared to the results reported by Jollow et al. (81). This may be a function of the greater dose (300 mg/kg, i.p.) administered to rats in this study.

CHAPTER VI

SUMMARY

The purpose of this study was to examine the effect of dietary sulfur on the hepatic metabolism and urinary excretion of acetaminophen in adult male rats. In this investigation, dietary inorganic sulfate did not appear to affect the hepatic activities of the glutathione S-transferases, sulfotransferase, and UDP-glucuronyltransferase or the concentration of reduced glutathione. In addition, the urinary excretion of acetaminophen sulfate was unaffected by the level of dietary inorganic sulfate, suggesting that the sulfation pathway was operating at full capacity given the drug dose used (300 mg/kg, i.p.). In an effort to compensate for the steady-state production of acetaminophen sulfate, the urinary excretion of free acetaminophen, acetaminophen glucuronide, and the mercapturic acid conjugate was increased at the lowest level of dietary inorganic sulfate (0.0072%).

In rats receiving acetaminophen, supplementation of diets with organic sulfur (cysteine + methionine) reduced hepatic glutathione S-transferase activity, increased hepatic glutathione levels, and decreased the urinary excretion of the glucuronide conjugate. Only the urinary excretion of the glucuronide conjugate of acetaminophen appeared to be sensitive to the type of dietary organic sulfur supplementation: the urinary excretion of acetaminophen glucuronide was greater in rats fed diets supplemented with cysteine than in rats fed diets supplemented with methionine.

Acetaminophen sulfate comprised most of the total dose excreted (32 - 50%), followed by acetaminophen glucuronide (20 - 38%), free acetaminophen (13 - 27%), and acetaminophen mercapturic acid (6 - 10%). In general, these values are in agreement with values reported in the literature.

The significance of this study is that it provides evidence that the hepatic metabolism and urinary excretion of acetaminophen is affected by sulfur status in the rat. The metabolism and excretion of acetaminophen by the rat appears to be more sensitive to the presence and type of organic sulfur in the diet than to the level of dietary inorganic sulfate.

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LIST OF REFERENCES

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APPENDICES

APPENDIX A

SAS PROGRAM USED TO GENERATE THE CONTRAST STATEMENTS IN THE ANALYSIS OF PART I DATA

```
DATA GSHT;
INPUT DIET 1-2 RAT 4 SO4LEV 6 SUPPLE 8 SPACLUN 10-14 WWTLUN 16-18
  SPACLIV 20-23 WWTLIV 25-28;
SPACLUN = SPACLUN + 0.9897;
WWTLUN = WWTLUN + 1.7452;
SPACLIV = SPACLIV + 1.9889;
WWTLIV = WWTLIV + 2.5057;
GRP = 2;
CARDS;
DATA ONE;
INPUT DIET 1-2 RAT 4 SO4LEV 6 SUPPLE 8 SPACLUN 10-14 WWTLUN 16-20
  SPACLIV 22-25 WWTLIV 27-30;
GRP = 1;
CARDS;
PROC SORT;
  BY DIET;
PROC PRINT;
PROC GLM;
  CLASSES SO4LEV SUPPLE;
  MODEL SPACLUN=SO4LEV SUPPLE SO4LEV*SUPPLE;
  LSMEANS SO4LEV SUPPLE SO4LEV*SUPPLE/STDERR;
  CONTRAST '.0072 VS .027'
  SO4LEV 1 -1 0;
  CONTRAST '.027 VS .427'
  SO4LEV 0 1 -1;
  CONTRAST 'SUPPLE VS NO SUPPLE'
  SUPPLE 1 -.5 -.5;
  CONTRAST 'CYS VS METH'
  SUPPLE 0 1 -1;
PROC GLM;
  CLASSES SO4LEV SUPPLE;
  MODEL WWTLUN=SO4LEV SUPPLE SO4LEV*SUPPLE;
  LSMEANS SO4LEV SUPPLE SO4LEV*SUPPLE/STDERR;
  CONTRAST '.0072 VS .027'
  SO4LEV 1 -1 0;
  CONTRAST '.027 VS .427'
  SO4LEV 0 1 -1;
  CONTRAST 'SUPPLE VS NO SUPPLE'
  SUPPLE 1 -.5 -.5;
  CONTRAST 'CYS VS METH'
  SUPPLE 0 1 -1;
```

```
PROC GLM;
  CLASSES SO4LEV SUPPLE;
  MODEL SPACLIV=SO4LEV SUPPLE SO4LEV*SUPPLE;
  LSMEANS SO4LEV SUPPLE SO4LEV*SUPPLE/STDERR;
  CONTRAST '.0072 VS .027'
  SO4LEV 1 -1 0;
  CONTRAST '.027 VS .427'
  SO4LEV 0 1 -1;
  CONTRAST 'SUPPLE VS NO SUPPLE'
  SUPPLE 1 -.5 -.5;
  CONTRAST 'CYS VS METH'
  SUPPLE 0 1 -1;
PROC GLM;
  CLASSES SO4LEV SUPPLE;
  MODEL WWTLIV=SO4LEV SUPPLE SO4LEV*SUPPLE;
  LSMEANS SO4LEV SUPPLE SO4LEV*SUPPLE/STDERR;
  CONTRAST '.0072 VS .027'
  SO4LEV 1 -1 0;
  CONTRAST '.027 VS .427'
  SO4LEV 0 1 -1;
  CONTRAST 'SUPPLE VS NO SUPPLE'
  SUPPLE 1 -.5 -.5;
  CONTRAST 'CYS VS METH'
  SUPPLE 0 1 -1;
```

APPENDIX B

SAS ANALYSIS OF COVARIANCE PROGRAM

```
DATA GSHST;
INPUT DIET 1-2 RAT 4 SO4LEV 6 SUPPLE 8 SPACLUN 10-14 WWTLUN 16-18
  SPACLIV 20-23 WWTLIV 25-28;
CARDS;
PROC SORT;
  BY DIET RAT;
PROC PRINT;
  TITLE RAW PHASE1 DATA;
PROC GLM;
  CLASSES DIET;
  MODEL SPACLUN WWTLUN SPACLIV WWTLIV=DIET RAT DIET*RAT/SOLUTION;
  LSMEANS DIET/STDERR;
  ESTIMATE 'DIET 4 MEAN AT RAT 1'
  INTERCEPT 1 DIET 1 0 0 0 0 0
  RAT 1;
  ESTIMATE 'DIET 5 MEAN AT RAT 1'
  INTERCEPT 1 DIET 0 1 0 0 0 0
  RAT 1;
  ESTIMATE 'DIET 6 MEAN AT RAT 1'
  INTERCEPT 1 DIET 0 0 1 0 0 0
  RAT 1;
  ESTIMATE 'DIET 7 MEAN AT RAT 1'
  INTERCEPT 1 DIET 0 0 0 1 0 0
  RAT 1;
  ESTIMATE 'DIET 8 MEAN AT RAT 1'
  INTERCEPT 1 DIET 0 0 0 0 1 0
  RAT 1;
  ESTIMATE 'DIET 9 MEAN AT RAT 1'
  INTERCEPT 1 DIET 0 0 0 0 0 1
  RAT 1;
  TITLE ANCOVA ON PHASE1 DATA;
```

APPENDIX C

ANALYSIS OF COVARIANCE SUMMARY FOR PHASE 1 DATA (WITH INTERACTION)

Dependent Variable	Source	Degrees of Freedom	Sums of Squares	F-Value	P-Value
SPACLUN	Diet	6	4.9938	1.06	0.4197
	Rat	1	13.8444	17.58	0.0004
	Diet*Rat	6	4.7919	1.01	0.4440
WWTLUN	Diet	6	4.6783	0.54	0.7723
	Rat	1	41.7605	28.87	0.0001
	Diet*Rat	6	4.0582	0.47	0.8241
SPACLIV	Diet	6	4.7196	1.34	0.2842
	Rat	1	69.2221	117.84	0.0001
	Diet*Rat	6	2.2615	0.64	0.6960
WWTLIV	Diet	6	28.5512	2.92	0.0314
	Rat	1	109.8755	67.38	0.0001
	Diet*Rat	6	14.3474	1.47	0.2375

APPENDIX D

SAS PROGRAM USED TO FIT A LINE TO EACH STANDARD CURVE IN THE ANALYSIS OF URINE SAMPLES BY HPLC

```
DATA CURVE;
INPUT STDCUR 1 CONC 3-5 RATIO 7-9;
CARDS;
PROC SORT;
  BY STDCUR;
PROC GLM;
  MODEL CONC=RATIO;
  BY STDCUR;
  OUTPUT OUT=NEW PREDICTED=YHAT RESIDUAL=RESID;
PROC PLOT DATA=NEW;
  PLOT YHAT*RESID;
```

VITA

Diane Helen Morris was born in Chattanooga, Tennessee, on September 27, 1949. She was graduated from Chattanooga High School in June 1967 and entered The University of Tennessee, Knoxville, the following September. In June 1971, she was awarded the Bachelor of Science degree in Home Economics by The University of Tennessee. The following year she worked as a dietetic intern at Lakeshore Mental Health Institute.

She returned to The University of Tennessee, Knoxville, in July 1977 to pursue graduate study. She by-passed the Master's degree and received the Doctor of Philosophy degree in Home Economics in June 1982. Her major was Nutrition Science; she had collateral areas of study in Biochemistry and Statistics. While at The University of Tennessee, the author held a teaching assistantship for two years and was a recipient of a General Foods Fellowship Award. She was president of Omicron Nu and a member of Phi Kappa Phi.

She is married to Larry Ross Kimball of Smithfield, North Carolina.