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I am submitting herewith a dissertation written by Mary Lelia Gilbert entitled "The Effect of Dietary Inorganic Sulfate on the Activity of Glucose-6-Phosphate Dehydrogenase in Rat Liver." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Home Economics.

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THE EFFECT OF DIETARY INORGANIC SULFATE ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN RAT LIVER

A Dissertation

Presented for the

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Degree

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Mary Lelia Gilbert

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ABSTRACT

The effect of the level of dietary inorganic sulfate ($SO_4^=$) on the activity of rat liver glucose-6-phosphate dehydrogenase (G6PDH), as measured by the appearance of NADPH, was investigated. Male rats of a Long-Evans, Wistar, Sprague Dawley mixed strain were fed 15% casein diets containing 0.0002% $SO_4^=$, 0.02% $SO_4^=$ (optimum level), or 0.42% $SO_4^=$, with total sulfur kept constant with either methionine or cysteine, or with constant cysteine. Preliminary tests using 350 to 450 g rats fed the test diets for periods of 17 or 22 days showed significantly higher ($P < 0.1$) enzyme activities at the 0.0002% $SO_4^=$ level than at the 0.02% $SO_4^=$ level, with no difference between the 0.02% level and the 0.42% level when total sulfur was kept constant with methionine supplementation. When cysteine was kept constant, no differences in enzyme activity between the diets were observed. If, instead of 350 g to 450 g rats, 150 g to 250 g rats were used, and the diets were fed for an average of 32 days, maximum enzyme activities were observed at the 0.02% level of $SO_4^=$, when total dietary sulfur was kept constant with methionine supplementation. When dietary sulfur was kept constant with cysteine supplementation, no significant differences in enzyme activity were observed and when cysteine

was kept constant, there was a significant increase ($P < 0.05$) from the low $SO_4^=$ level to the high level.

Since oxidized glutathione (GSSG) has been implicated in the control of G6PDH activity, GSSG levels were measured by a manometric technique in the supernatants of the livers of the 150 to 250 g rats. Also, the in vitro effect of physiological levels of GSSG on G6PDH activity was tested. When dietary sulfur was kept constant by methionine supplementation, GSSG levels were found to decrease significantly ($P < 0.05$) with increasing dietary $SO_4^=$ levels. In vitro, GSSG did not completely eliminate the differential effect of $SO_4^=$ on G6PDH activity. Thus, it was concluded that GSSG levels could only partially account for the decrease in G6PDH activity at $SO_4^=$ levels above the optimum and could not account for the decrease in activity at the low sulfate level. When dietary sulfur was kept constant with cysteine supplementation, a significant decrease ($P < 0.1$) in the GSSG level was observed at the high level of dietary sulfate only. When dietary cysteine was kept constant, there were no differences in GSSG levels, thus GSSG levels did not account for the G6PDH effect.

Glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-R) activities in the livers of 150 to 250 g rats were also measured. As the $SO_4^=$ level was decreased below the optimum level, GSH-Px and GSSG-R activities

decreased; however, above the optimum level of $SO_4^=$, GSSG-R activities continued to increase slightly but GSH-Px levels dropped, explaining the decrease in GSSG levels at a sulfate level above the optimum level.

The in vitro effects of physiological levels of $SO_4^=$ on the differential effect of dietary sulfate on G6PDH was also tested. Like GSSG, $SO_4^=$ only reduced the differential effect of sulfate when total sulfur was kept constant with methionine supplementation, but did not eliminate it. The increase in G6PDH activity with increasing dietary $SO_4^=$ was not affected by in vitro $SO_4^=$ when dietary cysteine was kept constant.

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CHAPTER I

INTRODUCTION

It was long accepted that all of the dietary sulfur requirements of the body could be satisfied by the sulfur-containing amino acids, methionine and cysteine. It is well documented that the sulfur atom of methionine can be transferred to serine, yielding a precursor of cysteine. The sulfur moiety of cysteine can subsequently be oxidized and removed, yielding sulfate ($SO_4^=$), pyruvate and ammonia. Thus, the sulfate requirements of the body could, theoretically, be supplied by methionine.

However, a series of studies providing evidence that dietary sulfate itself may affect various metabolic processes have been conducted in this laboratory. Investigations in this laboratory have shown that, in order to avoid metabolic stress, inorganic sulfate at a level of 0.02% must be included in the diet of a rat. This level was thus defined as the optimum level of dietary sulfate. Further investigations revealed that dietary sulfate could affect a number of enzymes involved in carbohydrate and lipid metabolism. Among these enzymes that were found to be affected by dietary sulfate was acetyl CoA carboxylase, the controlling enzyme of fatty acid synthesis. When total dietary sulfur was kept constant by varying the methionine levels, or when total

dietary cysteine was kept constant, significantly higher ($P < 0.01$) acetyl CoA carboxylase activities were observed at the 0.02% level of dietary sulfate than when higher or lower levels of dietary sulfate were fed. This evidence suggests that there is a higher rate of fatty acid synthesis at the optimum level of dietary sulfate than at levels above or below the optimum. Since fatty acid synthesis requires NADPH as a reducing equivalent, a higher rate of fatty acid synthesis would logically require an increase in the production of NADPH. However, there was not an increase in malic enzyme activity at the optimum level of dietary sulfate and isocitrate dehydrogenase has not been found to fluctuate with fatty acid synthesis, these enzymes being two of the four that generate NADPH. Therefore, since the reducing equivalents required for fatty acid biosynthesis did not appear to be supplied by the activity of these enzymes at the optimum sulfate level, the present study was designed to determine if the reducing equivalents required for the increased fatty acid synthesis were supplied by glucose-6-phosphate dehydrogenase. The activity of 6-phosphogluconate dehydrogenase, the only other supplier of NADPH, depends on glucose-6-phosphate dehydrogenase for its substrate; therefore, its activity was not measured.

CHAPTER II

REVIEW OF LITERATURE

I. DIETARY SULFATE EFFECTS

Methionine was long accepted as the body's only essential source of sulfur (1) and thus of organic sulfate. Sulfate is needed by the body for the biosynthesis of gastrin II, mucopolysaccharides, thiosulfate, sulfolipids, heparin and bile salts (2). Cysteine can also supply sulfate ($SO_4^=$) for the synthesis of these compounds and cysteine has been shown to spare dietary methionine.

A series of studies providing evidence that dietary sulfate itself may affect various metabolic processes have been conducted in this laboratory. These studies followed up an initial observation that rats fed diets with variable sulfate/sulfur ratios showed different responses to avitaminosis E (2).[1] Vitamin E deficient rats appeared to be less effective in converting ^{35}S-cysteine to $^{35}SO_4^=$ than vitamin E sufficient rats. In vitro, sulfate was shown to inhibit the oxidation of cysteine sulfur to sulfate (2). This effect of dietary sulfate on cysteine desulfuration was

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[1]Smith, J. T., & Pendergrass, B. J. (1962) A relationship between vitamin E and the sulfation of mucopolysaccharides and cellular lipoproteins. Fed. Proc. 21, 170.

further supported by a study showing the distribution of radioactivity in the feces, blood and mucopolysaccharides from rib cartilage of rats fed diets containing ^{35}S-methionine and $Ca^{35}SO_4^=$ (2, 4, 5). Three conclusions were drawn from the data obtained in this study:

1. Sulfate ions from inorganic sources contribute as much to the metabolic pool as does endogenous sulfate oxidized from organic sources.
2. Low levels of dietary inorganic sulfate enhance the absorption of methionine.
3. High levels of dietary inorganic sulfate result in a better utilization of sulfate than diets high in neutral sulfur.

In fact, even 1.04% of methionine in a diet low in sulfate was not sufficient to supply the endogenous sulfate provided by a diet containing 0.1% $SO_4^=$.

It was hypothesized that the inefficient oxidation of neutral sulfur to sulfate was due to an increased conversion of cysteine to taurine. An increase in urinary taurine was seen in rats fed diets containing 0.0002% of $SO_4^=$ as compared to those containing 0.02% of $SO_4^=$ (2, 6). The glycocholate: taurocholate ratio of the small intestinal contents of rats did not support the theory that urinary taurine levels would reflect the availability of taurine for cholate conjugation unless the change was large (2, 7). Whittle et al. (8)

concluded that the formation of taurine conjugated bile salts is not completely controlled by the supply of taurine. However, in vitro, cholate conjugation studies and serum cholesterol measurements supported the concept that $SO_4^=$ and sulfur-containing amino acids may play a regulatory role in cholesterol metabolism (2).

The variable incorporation of sulfate into rib cartilage mucopolysaccharides in rats fed diets with variable sulfate levels suggested an investigation of the effect of dietary inorganic sulfate on the strength of the aorta. A decrease in the collagen content of the aorta and a decreased breaking strength of the aorta were found in rats fed diets containing 0.0002% of $SO_4^=$ as compared to those fed diets containing 0.02% of $SO_4^=$ when total dietary sulfur was kept constant (2, 9, 10).

The optimum level of sulfate in the rat diet was determined by measuring $^{14}CO_2$ expiration following a dose of ^{14}C-methionine (11). There was a minimum expiration of $^{14}CO_2$ by rats fed diets containing 0.02% of sulfate. The minimum expiration of $^{14}CO_2$ was assumed to indicate maximal sparing of the carbon skeleton of cysteine in rats fed diets containing 0.02% $SO_4^=$. An increase in $^{14}CO_2$ expiration with higher sulfate levels indicated that sulfate must play a regulatory role in metabolism other than that related to the metabolism of the sulfur-containing amino acids (2).

Dietary sulfate has been shown to affect the activity of a number of enzymes involved in carbohydrate and lipid metabolism. Some of these include phosphoenolpyruvate carboxykinase, propionyl CoA carboxylase, malic enzyme, citrate cleavage enzyme and acetyl CoA carboxylase (2). The greater activity of phosphoenolpyruvate (PEP) carboxykinase in rats fed diets containing 0.02% of $SO_4^=$ is thought to explain the increased storage of the cysteine carbon skeleton as glycogen, as PEP carboxykinase is the controlling enzyme of gluconeogenesis. Propionyl CoA carboxylase activity was lowest in rats fed diets containing 0.02% of $SO_4^=$.[2] Propionyl CoA carboxylase activity may be a reflection of a decrease in the metabolism of the carbon skeleton of methionine (2). Acetyl CoA carboxylase activity was highest in rats fed diets containing 0.42% $SO_4^=$,[3] but the activity of malic enzyme did not reflect an increased supply of NADPH required for the increased fatty acid synthesis accompanying the higher acetyl CoA carboxylase activity (2).

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[2]White, J. V. (1975) Effect of sulfur nutrition on propionyl CoA carboxylase activity in rat liver. Unpublished Doctoral Dissertation, The University of Tennessee, Knoxville.

[3]Bittle, J. B. (1977) Effect of sulfur nutrition on the activity of acetyl coenzyme A carboxylase, citrate cleavage enzyme, and malic enzyme in rat liver. Unpublished Doctoral Dissertation, The University of Tennessee, Knoxville.

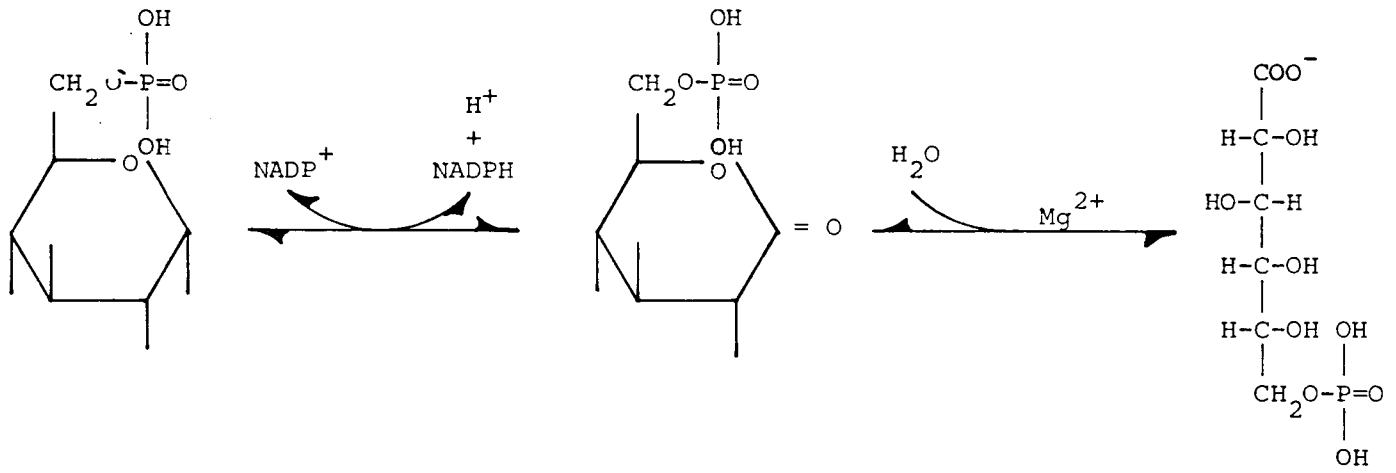
II. GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Reaction Catalyzed

Glucose-6-phosphate dehydrogenase (d-glucose-6-phosphate: NADP 1-oxidoreductase, G6PDH, E.C. 1.1.1.49) catalyzes the oxidation of d-glucose-6-phosphate (G6P) to form d-glucone-δ-lactone 6-phosphate (12) by the reaction shown in Figure 1. This reaction requires the cofactor, nicotine adenine dinucleotide phosphate (NADP), which is consequently reduced to NADPH. Glucone-δ-lactone 6-phosphate is spontaneously converted to 6-phosphogluconate (6PG) (13); thus, 6PG is sometimes referred to as the product of the G6PDH catalyzed reaction (14).

Substrate Specificity

G6PDH is highly specific for G6P (12), and NADP (13); however, Anderson, Horne and Nordlie (15-17) have observed a relatively slow oxidation of glucose, catalyzed by an enzyme identified as G6PDH by thermal, ionic strength and differential ion effects, as well as electrophoretic properties (16). Glucose dehydrogenase activity normally approximates 1% of the G6PDH activity (17). The enzyme is nonspecific with respect to stereoconfiguration of carbon atom one (13); thus, α or β isomers of G6P can be utilized as substrates.



D-Glucose-6-phosphate D-Glucose-δ-lactone-6-P 6-Phospho-D-glucose

Figure 1. Reaction catalyzed by glucose-6-phosphate dehydrogenase.

Properties

Molecular Properties

The purified enzyme, according to Holten (18), exists in at least three different dimeric forms of molecular weight 130,000, which account for about 90% of the total activity of the enzyme. These dimers can aggregate to form tetrameric and hexameric forms which account for most of the remaining activity. These active forms, consisting of inactive sub-units of molecular weight 64,000, are immunologically identical. The purified enzyme shows three to four sub-bands upon electrophoresis on polyacrylamide gels. These observations are similar to those reported for mouse liver enzyme by Hizi and Yagil (19).

Vesell et al. (20), quoting Shaw, reported that the differences in G6PDH isozyme patterns of mouse liver and kidney disappear when tissue homogenates are dialyzed prior to electrophoresis. They concluded that some dialyzable substance in liver altered the G6PDH isozyme pattern to suggest erroneously the existence in liver of a biochemically and genetically distinct isozyme from that in kidney. The G6PDH gene is located on the x-chromosome, thus there is a single copy of the gene in males (13).

Noltman et al. (13) stated that the NADP-free apoenzyme appeared to be a monomer and the NADP-enzyme was a dimer.

The enzyme may contain Zn as a functional component, or it may require it for biosynthesis (13).

Kinetic Properties

According to Holten et al. (21), equilibrium dialysis indicates that rat liver G6PDH binds two molecules of NADP per subunit with a dissociation constant of 6.0×10^{-7} M. The NADP-free enzyme will not bind G6P, indicating a compulsory order of substrate binding. Anderson et al. (16) reported that the K_m value for NADP was not influenced by the G6P present and that the K_m value for G6P was independent of the NADP concentration. There is, therefore, no competition for binding sites between the two substrates.

Greenbaum et al. (22) quoted estimates of the K_m values for G6PDH ranging from 1.3×10^{-5} to 3.9×10^{-5}. Noltman et al. (13) reported a K_m value of 1.3×10^{-5} for rat liver G6PDH with respect to both NADP and G6P (pH 7.6, glycylglycine, 20°). Anderson et al. (16) observed that, at pH 8.0, a double-reciprocal plot in which G6P concentration was varied had a bimodal nature, resulting in two K_m values for G6PDH. They found a K_m of 8.0×10^{-5} for high concentrations of G6P (greater than 1.0×10^{-4}m G6P) and a K_m of 3.4×10^{-5} for low G6P concentrations (Tris-Cl buffer). This effect was not seen at pH 7.3, nor when glucose was the substrate used. Hizi et al. (19), and Noltman et al. (13), also referred to the bimodal nature of the enzyme.

Thermodynamic Properties

G6PDH catalyzes an essentially irreversible reaction (13, 23), as almost complete conversion of G6P to 6PG is thermodynamically favored (24). The initial reaction is reversible with the δ-lactone of 6PG being capable of initiating the reverse reaction (13). However, the rapid hydrolysis of the lactone to 6PG results in a large free energy change, making the reconversion of 6PG to G6P almost impossible (25).

pH Optimum

Hizi and Yagil (19) reported a broad region of maximal activity between pH 7.5 and 9.5 for G6PDH in mouse liver.

Circadian Rhythm

Yagil and Hizi (26) observed a circadian rhythm in mouse liver G6PDH with maximal activity at midnight and minimum activity at midday.

Distribution

G6PDH activity has been demonstrated in almost all animal tissues and micro-organisms (14). Blood cells, adipose tissue and lactating mammary gland tissue are especially rich sources of the enzyme. Less occurs in liver, pancreas, kidney, lung, brain and gastric mucosa,

while only traces are found in skeletal and heart muscle and virtually none in serum. Some tumors contain a high activity of the enzyme.

The G6PDH activity of the cell is confined exclusively to the cytoplasm, as are the phosphorylated sugars (22).

Historical Background

G6PDH has a distinguished history in that it was not only the first dehydrogenase to be highly purified (by Negelein and Haas in the laboratory of Otto Warburg in 1935), but it was also the enzyme which led Warburg to the identification of nicotinamide as the reactive component of the coenzyme, NADP (24). Warburg names the G6PDH catalyzed reaction the Zwischenferment reaction (13).

Control of the Pentose Phosphate Pathway

The pentose phosphate pathway, also known as the hexose monophosphate shunt, is thought to be controlled by the rate of G6PDH activity. Greenbaum et al. (22) demonstrated by a comparison of equilibrium constants, mass action ratios and metabolite profiles, that G6PDH was the control point in the oxidative portion of the pentose phosphate pathway. Weber et al. (23) demonstrated that the activity of G6PDH at substrate saturation levels of G6P and at tissue concentration

levels was one-half to one-third that of 6PG dehydrogenase. They also showed that the tissue concentration of 6PG was about 5% that of G6P in rat liver. These data give further evidence of the controlling action of G6PDH.

Fine Control of G6PDH Activity

Introduction

The pentose phosphate pathway has two major functions (23, 27):

1. To supply NADPH to be used as a reducing agent in fatty acid synthesis, in steroidogenesis, for reduction of glutathione and methemoglobin and for H_2O_2 production in phagocytes.
2. To supply ribose phosphate for the synthesis of nucleic acids.

Fluctuations in the rates of these processes can occur throughout the day, requiring a rapid adjustment—or fine control—of pentose phosphate pathway activity. As G6PDH is the controlling enzyme in this pathway, its rate of activity must be finely controlled by factors related to the above processes. Fine control is usually accomplished by allosteric effectors (24), by substrate limitation, by product removal, or by a combination of two or more of these mechanisms. It

has been suggested that the G6P level is a major factor in determining the rate of its own oxidation (15). However, Greenbaum et al. (22), Weber et al. (23) and Eggleston et al. (25), questioned this hypothesis on the grounds that the cellular concentration of this metabolite exceeds the Km by more than three-fold. Also, G6P is at a branch point in glucose metabolism. Weber et al. (23) quoted estimates of the contribution of the pentose phosphate pathway to total glucose utilization ranging from 1% to 30%. Thus, since the pentose phosphate pathway is important for reasons other than the metabolism of glucose, it is reasonable to assume that control of G6PDH is not achieved by substrate limitation. The spontaneous removal of the product, glucono-lactone, the absence of an inhibitory effect of 6PG (24) and the large difference between steady state conditions and thermodynamic equilibrium (25) imply that allosteric effectors must play a major role in the regulation of G6PDH activity. Some metabolites that are related to the primary functions of the pentose phosphate pathway and that have been shown to affect G6PDH activity include NADPH, oxidized glutathione (GSSG), nucleotide triphosphates, fatty acids and steroids. Certain anions have also been shown to have an effect on the enzyme, including orthophosphate, sulfate and bicarbonate.

NADP/NADPH

According to Krebs and Eggleston (24), Warburg, Negelein and Haas observed that NADPH inhibited G6PDH activity and this inhibition was not simple end-product inhibition; i.e., not a matter of the stoppage of a reaction because equilibrium has been reached. The observation that the inhibition by NADPH was competitive with NADP (25) and that NADP/NADPH ratios varied under the same conditions that altered G6PDH activities (22), led Holten et al. (21), and others, to conclude that the NADP/NADPH ratio controls the rate of G6PDH activity. Schachet et al. (28) observed a controlling effect of the reducing charge, equal to [NADP]/[NADP] + [NADPH] on G6PDH activity in bovine adrenal tissue. According to Holten et al. (21), a rapid rate of fatty acid synthesis could utilize NADPH rapidly and increase the NADP/NADPH ratio. This would rapidly increase the activity of G6PDH and tend to restore the NADP/NADPH ratio back to its normal value. Greenbaum et al. (22) have observed changes in the NADP/NADPH ratio with changes in nutritional state. They observed a high ratio in rats on lipogenic diets corresponding to a 98% inhibition of G6PDH.

Oxidized Glutathione

Eggleston and Krebs (24, 25) noted that the physiological ratios of free NADPH/free NADP would completely inhibit

G6PDH. They found that NADPH/NADP ratios in vitro above eight completely inhibited the enzyme, whereas Veech et al. (29) reported in vivo NADPH/NADP ratios of about 100. Also, concentrations of NADPH amounting to about one-third of the concentration of total NADPH in the liver were strongly inhibitory in vitro (24). Eggleston and Krebs (24, 25) concluded that control of the enzyme in vivo must be a matter of de-inhibition by some cell constituent. They tested over 100 substances (including amino acids, phosphates, coenzymes, B vitamins, substrates and intermediates of respiration and glycolysis, hormones and purine nucleotides and nucleosides) and found only two that had significant effects—AMP and GSSG. Of these, only GSSG was effective at physiological concentrations (0.0123 to 0.2mM). However, the de-inhibition of G6PDH by GSSG in vivo has not been proven because of a lack of reliable information on the tissue concentration of GSSG. Wendell (30) calculated the intracellular concentration of GSSG in rat liver to be about 0.06mM from the data of Guntherberg and Rapoport. The effect of GSSG was not due to the removal of NADPH by the GSSG reductase (GSSG-R) reaction, as controls indicated that there was no measurable GSSG-R activity when the concentration of GSSG was below 0.2mM. Also, the presence of Zn^{++} (33.3μM) which completely inhibits GSSG-R, did not alter the effect. Dialysis of the homogenates for 24 hours eliminated the de-inhibition by GSSG, as did

purification of the enzyme. Thus, it was concluded that the de-inhibition by GSSG required a cofactor.

The rate of the glutathione peroxidase (GSH-Px) catalyzed reaction depends on the availability of substances which, on oxidation by O_2, can form H_2O_2, such as hypoxanthine or uric acid (24). When physiological concentrations of these substances were added to the reaction media in the presence of reduced glutathione, de-inhibition of G6PDH was again observed (24). This apparently resulted from the formation of GSSG via the peroxidase reaction. H_2O_2 has also been shown to stimulate the pentose phosphate pathway (31).

Evidence has been presented by Isaacs et al. (32) for the concept that the formation of mixed disulfides of proteins and glutathione is a mechanism for the maintenance of a disulfide-sulfhydryl ratio such that the integrity of membranes is maintained during oxidative and reductive stresses on the hepatic cell. Glutathione, in the reduced (GSH) or oxidized form, can enter into disulfide-sulfhydryl exchange reactions with disulfide or sulfhydryl groups on proteins, resulting in the formation of mixed disulfides:

$$GSH + ProtSSProt \longleftrightarrow GSSProt + ProtSH$$

$$GSSG + ProtSH \longleftrightarrow GSSProt + GSH$$

G6PDH has a diurnal variation with activity highest during periods of maximal mixed disulfide formation and lowest

during the period of minimal mixed disulfide formation. The evidence presented suggests that G6PDH may be one of the cytosol proteins which physiologically forms glutathione mixed disulfides.

There is some heat labile, large molecular weight, factor that is needed for the formation of glutathione mixed disulfides selectively with cytosol proteins. Isaacs et al. (32) refers to a thiol transferase that has recently been isolated and characterized from rat liver cytosol, which catalyzes the reversible formation of mixed disulfides. This observation may be related to the cofactor requiring de-inhibition of G6PDH by GSSG.

Watanabe et al. (33) observed that G6PDH is converted into various forms by interaction with GSH. These forms had similar molecular weights but different net charges. They concluded that at least two readily reactive but catalytically nonessential sulfhydryl groups are present per dimer and are involved in the interconversion of the forms. The observation of Watanabe et al. (33) further supports the theory of Isaacs et al. (32) that G6PDH forms glutathione mixed disulfides.

Nucleosides and Anions

In the liver cell, ribose-5-phosphate (R5P) is generated via the pentose phosphate pathway. R5P may be utilized for recycling into glycolysis through the transketolase and transaldolase reactions or it may be utilized as a precursor

for the synthesis of phosphoribosyl pyrophosphate (PRPP) by the enzyme, PRPP synthetase. Since R5P plays a major role in purine biosynthesis, increased utilization of purines for DNA and RNA synthesis, as in cellular replication or for nucleoside phosphates used in energy metabolism, would increase the requirement for R5P synthesis, resulting in greater pentose phosphate pathway activity. A decreased utilization of R5P would result in a temporary enlargement of the pentose phosphate pool, and pentose would be recycled into glycolysis (23), through the transketolase and transaldolase reactions. It has been demonstrated that ATP and other nucleoside phosphates inhibit G6PDH activity (15, 16, 34), which may account for the recycling of R5P when it is no longer needed for purine biosynthesis. This inhibition was much more pronounced at neutral pH values than at higher pH values and the inhibition could be relieved competitively with G6P but not with NADP. Mg^{++} strongly diminished the inhibition by ATP (34). Horne et al. (15), observed a rather broad specificity of inhibition of G6PDH activity with respect to nucleoside 5'-triphosphates and nucleoside 5'-diphosphates. Nucleoside triphosphates were more effective inhibitors than nucleoside diphosphates, and purine nucleosides were more potent inhibitors than were the pyrimidine nucleosides. R5P was relatively ineffective, as were adenine and adenosine.

Anderson, Horne and Nordlie (15-17) confirmed previous observations that orthophosphate ($HPO_4^=$), sulfate ($SO_4^=$) and bicarbonate (HCO_3^-) inhibit G6PDH activity. They found this inhibition to be competitive with G6P but not with NADP. Maximal inhibition was observed at pH 7.3. Inhibition of G6PDH by all three anions, singly or in combinations of two or three, was of the "common-site" type. They also presented evidence that there was a common binding site between ATP and these ions. $HPO_4^=$, $SO_4^=$ and HCO_3^- stimulated the glucose dehydrogenase activity of the enzyme, whereas ATP inhibited its glucose dehydrogenase activity.

Horne et al. (15) presented a hypothetical mechanism incorporating these observations and the view that an enzyme's active site is flexible and that the substrate is able to induce a change in the shape of this active site, resulting in a catalytically active fit of enzyme and substrate. A highly schematic diagram of this mechanism adapted from Horne et al. (15) is presented in Figure 2. According to their hypothesis:

> The free enzyme with binding sites A, B and C for glucose-6-P is depicted in I. NADP is postulated to bind initially to this enzyme to produce the binary complex II. As shown in III, glucose-6-P may then bind to this enzyme NADP complex in the absence of an anion, and induce a rearrangement of the active site such that NADP and the hemiacetal of the sugar phosphate substrate would be spacially oriented properly so that the reaction may ensue. Glucose by itself would be unable to produce such a "flexation" in the active site, as indicated in

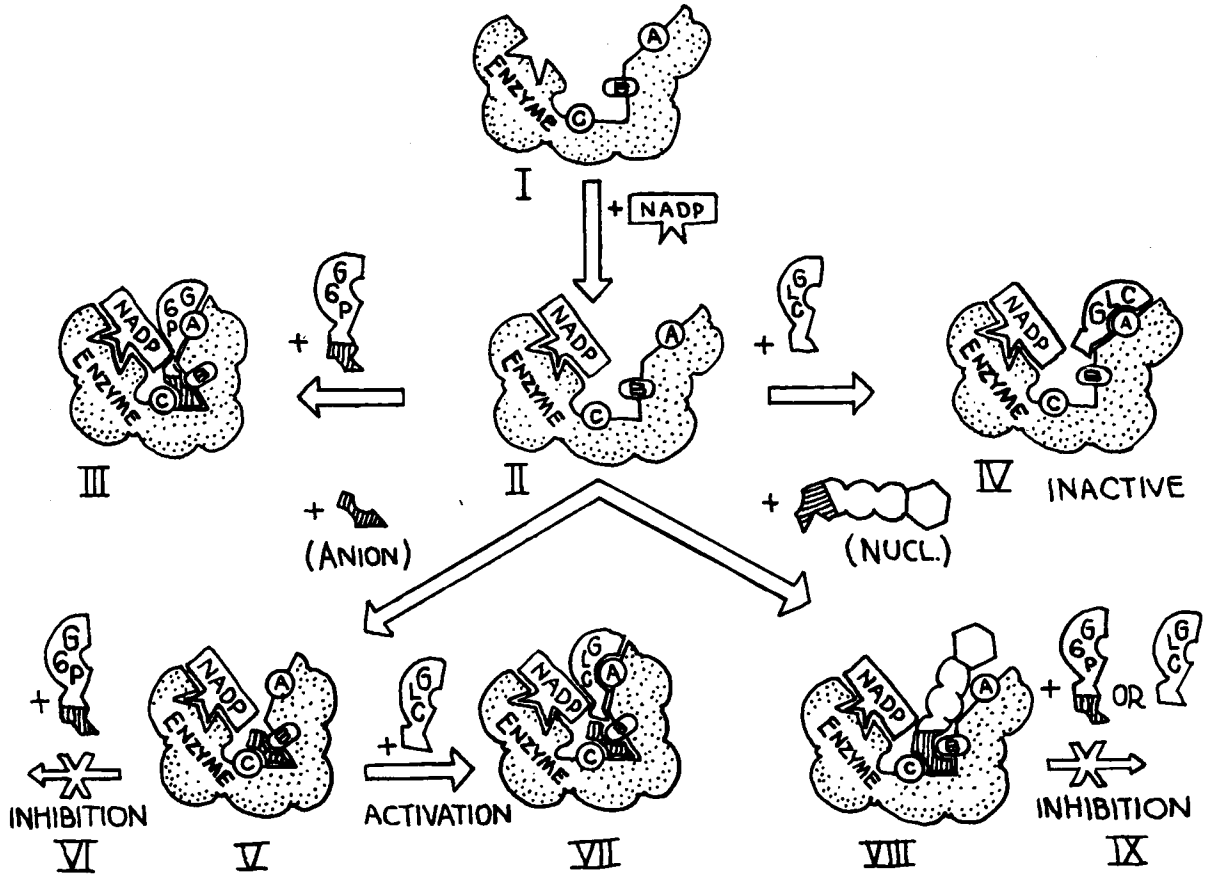


Figure 2. Postulated mechanism of action of nucleoside 5'-triphosphates or diphosphates and inorganic anions in selectively controlling yeast glucose dehydrogenase and glucose-6-P dehydrogenase activities. (Adapted from Horne et al. (15).)

> IV; however, the addition of anion, binding to hypothetical sites B and C, could transpose the enzyme NADP complex into the "flexed" state indicated by V. . . .

The anion would compete with G6P for binding sites B and C (VI) but would enhance the glucose dehydrogenase activity of the enzyme (VII), explaining the differential effect of anions on G6PDH activity and its glucose dehydrogenase activity. The competition between G6P, glucose and nucleoside phosphates is indicated by pathway II⟶ VIII. If the nucleoside phosphate binds to the active site for G6P and glucose, then the enzyme cannot react with these two substrates.

Horne et al. (15) further hypothesized:

> Inhibitions by nucleoside triphosphates and diphosphates, as well as by smaller anions, may constitute an effective mechanism for limiting G6P removal via direct oxidation, thus, possibly providing for the maintenance of G6P for glycogen synthesis. Further, it would seem reasonable from the present studies that in the presence of high concentrations of glucose, reversal by certain small anions of these nucleotide-effected inhibitions of glucose dehydrogenase and reinforcement by these same anions of the inhibition by nucleotides of G6PDH activity, could provide a mechanism for continued direct NADPH generation even under metabolic conditions in which G6P oxidation is rigorously controlled.

Fatty Acids

Long-chain acyl CoA has a feed-back inhibition on lipid synthesis at acetyl CoA carboxylase and may, thus, indirectly control the flux of glucose through the pentose phosphate

pathway. A more direct action of long-chain acyl CoA's on G6PDH has been postulated. While the CoA derivatives may be important effector molecules in the conditions that depress glucose utilization, it would not seem that changes in these metabolites could adequately explain the sharp rise in lipid synthesis and flux through the pentose phosphate pathway in diabetic rats treated with insulin or in starved rats refed a high carbohydrate diet (22). (Further discussion on this subject will follow.)

Coarse Control of G6PDH Activity

Dietary Effects

Quantitatively, the main NADPH-consuming process is the synthesis of fatty acids from carbohydrates where two molecules of NADPH are used for each acetyl CoA added to a fatty acid chain. Whether fatty acids are synthesized and, thus, whether NADPH is needed, depends on the presence in the diet of excess carbohydrate. In response to refeeding a high carbohydrate diet after a 48 hour fast (37, 38) or a chronic fast (39), there is a large increase in G6PDH activity following a 12 hour lag with the peak being reached 3 days after refeeding began (39). This increase constitutes an overshoot of the normal enzyme activity levels. De novo RNA synthesis is required for the overshoot but not for the recovery of normal levels of enzyme activity (40). This

overshoot is accompanied by super-normal levels of fatty acid synthesis in the liver (40); however, whereas a diet containing 4% of protein failed to stimulate an overshoot in G6PDH activity upon refeeding, the dietary protein had no effect upon the liver lipid concentration (39). This observation was interpreted as evidence that there is no direct association between the pentose phosphate pathway and lipogenesis.

Pentose phosphate pathway activity is very low in physiological states associated with carbohydrate deprivation such as diabetes, starvation or starvation followed by a high fat diet (22, 23, 36, 40). G6PDH activity is low in these physiological states (23, 40) and the NADPH/NADP ratio is high (22).

Several other dietary alterations have been shown to have an effect on G6PDH activity. Diets high in protein result in higher G6PDH activities and diets free of protein result in low G6PDH activities. However, in the latter case, food intake decreases, thus, the protein-free diet may affect the enzyme activity indirectly by altering carbohydrate and fat intake (40). Meal feeding rats also alters G6PDH activity and lipogenic activity. Following an initial drop in activity, the lipogenic activity increases to above normal values after 7 days and G6PDH activity increases to above control values after 9 days. This observation implies

that, although increased activity of G6PDH is not essential for the initial increase in fatty acid synthesis, it reflects the increased demand for NADPH to support fatty acid synthesis (41).

Diabetes and Insulin Effects

Administration of insulin to the alloxan diabetic rat decreases the NADPH/NADP ratio to normal (22) and induces G6PDH in a hormone dose-dependent fashion (42). This insulin induced increase in the enzyme level was blocked by administration of small doses of actinomycin (42). In alloxan diabetic rats, G6PDH activity is lower than normal. Also, the decreased activity of G6PDH observed in starvation was accompanied by decreased plasma insulin levels (23). Refeeding starved alloxan diabetic rats did not result in an overshoot in G6PDH levels, but did result in an increase back to the levels of control alloxan diabetic rats. However, if insulin were administered, refeeding produced an overshoot (42).

These observations imply that insulin is involved in the regulation of G6PDH activity. Based on their observation that anti-insulin serum prevented the increase in G6PDH levels upon refeeding a high carbohydrate diet to fasted animals, Weber et al. (23) proposed that insulin is the sole inducer of the enzyme. However, Rudack et al. (35) concluded

from their studies that insulin did not play a direct role in G6PDH regulation. They observed that rats given insulin were eating more than the corresponding controls and that there was a correlation between the G6PDH level and the amount of carbohydrate consumed. They also observed that fructose feeding had the same effect on G6PDH levels as did glucose feeding, which would not be expected if insulin were the sole inducer of the enzyme. Finally, they observed that the amount of insulin required to produce a significant increase in the enzyme was greater than that which could reasonably be called a physiological dose.

Hormonal Effects

Induction of G6PDH appears to result from a complex interaction of diet and hormones. The increased activity of G6PDH upon refeeding a diet high in carbohydrate could be abolished by several hormonal manipulations. Tepperman and Tepperman (43) observed a strikingly diminished response of the enzyme to refeeding in adrenalectomized rats. They found that treatment of these rats with somatrotropin, cortisone and thyroid hormone individually and in pairs failed to restore the induction of G6PDH in response to refeeding, but a combination of the three did restore the induction. However, there was evidence that this effect was due to a hormonal influence on the uptake and absorption

of carbohydrates rather than a direct effect on the enzyme. In contrast, Berdanier et al. (44) found that cortisol did restore the adaptive response of adrenalectomized rats to meal-feeding when fed a 65% glucose diet and that glucocorticoids increased lipogenesis, as well as hepatic G6PDH activity. Rudack et al. (35) showed that adenosine 3',5'-monophosphate (c-AMP), dibutyryl c-AMP and glucagon all prevented the induction of G6PDH by high carbohydrate diets without decreasing the amount of diet consumed. However, a direct effect of c-AMP on G6PDH has not been proven. Other hormones that have been shown to alter the response of this enzyme in liver to dietary changes are dehydroepiandrosterone (40) and 17β-estradiol (35).

Proposed Mechanisms of Adaptation

The adaptive responses of G6PDH to altered nutritional states may result from a combination of enzyme induction and altered NADP/NADPH ratios (22). Holten et al. (21) estimated that a combination of these two adaptive mechanisms could produce 30- to 50-fold changes in the capacity of the pentose phosphate pathway enzymes to produce NADPH during alterations in the nutritional state of the animal. There is disagreement in the literature concerning the mechanism by which the enzyme is induced. It has been generally accepted that induction and repression of G6PDH resulting from changes

in physiological states are accompanied by changes in the rate of synthesis or degradation of the enzyme. This assumption is supported by the relatively long period required for adjustment of enzyme activity to dietary changes as well as the effect of inhibitors of protein and RNA synthesis on induction. Also, Rudack et al. (35)—following the method of Berlin and Schimke for determining the half-life for the degradation in vivo of the enzyme and calculating steady state levels from the rate constants for enzyme synthesis and degradation—determined that increases in G6PDH activity upon refeeding a diet high in carbohydrate were due to increases in the rate of synthesis of the enzyme. Yagil and Hizi (46) questioned the validity of this conclusion, proposing that the same results could be obtained if activation of an existing enzyme were taking place. Yagil and Hizi (46), and Kelley et al. (47), determined—using immunoprecipitation procedures—that induction and repression of G6PDH by dietary manipulation were not accompanied by changes in the amount of enzyme protein. Yagil and Hizi concluded that an altered form of the enzyme, either completely or partially inactive, exists in livers of animals with repressed enzyme activity. They postulated that, during induction, these molecules are transformed to the more active form by some unknown mechanism. Based on the

blockage of induction by cyclohexamide and on the time required for induction to take place, Kelley et al. (47) rejected the theory that induction of G6PDH could be due to activation of an inactive enzyme. They proposed, alternatively, that the enzyme is continually being synthesized at a constant rate in the active form and that fed metabolites irreversibly inactivate the enzyme. This postulation explains the effect of dietary protein, of cyclohexamide and of the time delay in that protein synthesis is continually required. It explains the effect of starvation and fat feeding, in that fat metabolism increases under these conditions and it explains the overshoot in that very little fat will be metabolized initially upon refeeding a high carbohydrate diet. This hypothesis is supported by the observation of Eger-Neufeldt et al. (48) that long-chain acyl CoA inactivated G6PDH in vitro.

III. REGULATION OF GSSG LEVELS

Introduction

Oxidized glutathione (GSSG) has been implicated as an important factor in the regulation of G6PDH activity (24, 25). Tissue levels of GSSG are controlled primarily by glutathione reductase (GSSG-R) and glutathione peroxidase

(GSH-Px). However, certain glutathione disulfide transhydrogenases, as well as nonenzymatic oxidation catalyzed by Fe^{2+}, Cu^{2+}, Mn^{2+} or dehydroascorbic acid may also play a role in the regulation of the GSH/GSSG ratio (25). The sulfhydryl-proteins may also play a role in the regulation of free GSSG levels (32). GSSG levels constitute about 2% of the total glutathione and changes in the NADP/NADPH ratio do not alter the GSSG/GSH ratio (30).

The regulation of GSSG is associated with an important antioxidant system. A schematic representation of this system, adapted from Kimball et al. (49), is shown in Figure 3. GSH-Px reduces toxic lipid peroxides to their corresponding, less toxic, hydroxy-fatty acids, utilizing GSH as a cofactor, thus protecting sensitive membrane lipids from oxidation (31). GSSG-R replenishes GSH by reducing GSSG at the expense of NADPH. NADPH-generating enzymes are thus required to provide reducing equivalents to GSSG-R. These enzymes include G6PDH, 6PGDH, malate dehydrogenase and isocitrate dehydrogenase. Analysis of lung tissue from rats exposed to 90% oxygen for 7 days showed a 317% increase in GSH-Px activity, a 175% increase in GSSG-R activity, a 413% increase in G6PDH activity, and a 195% increase in GSH levels over those of the controls (49).

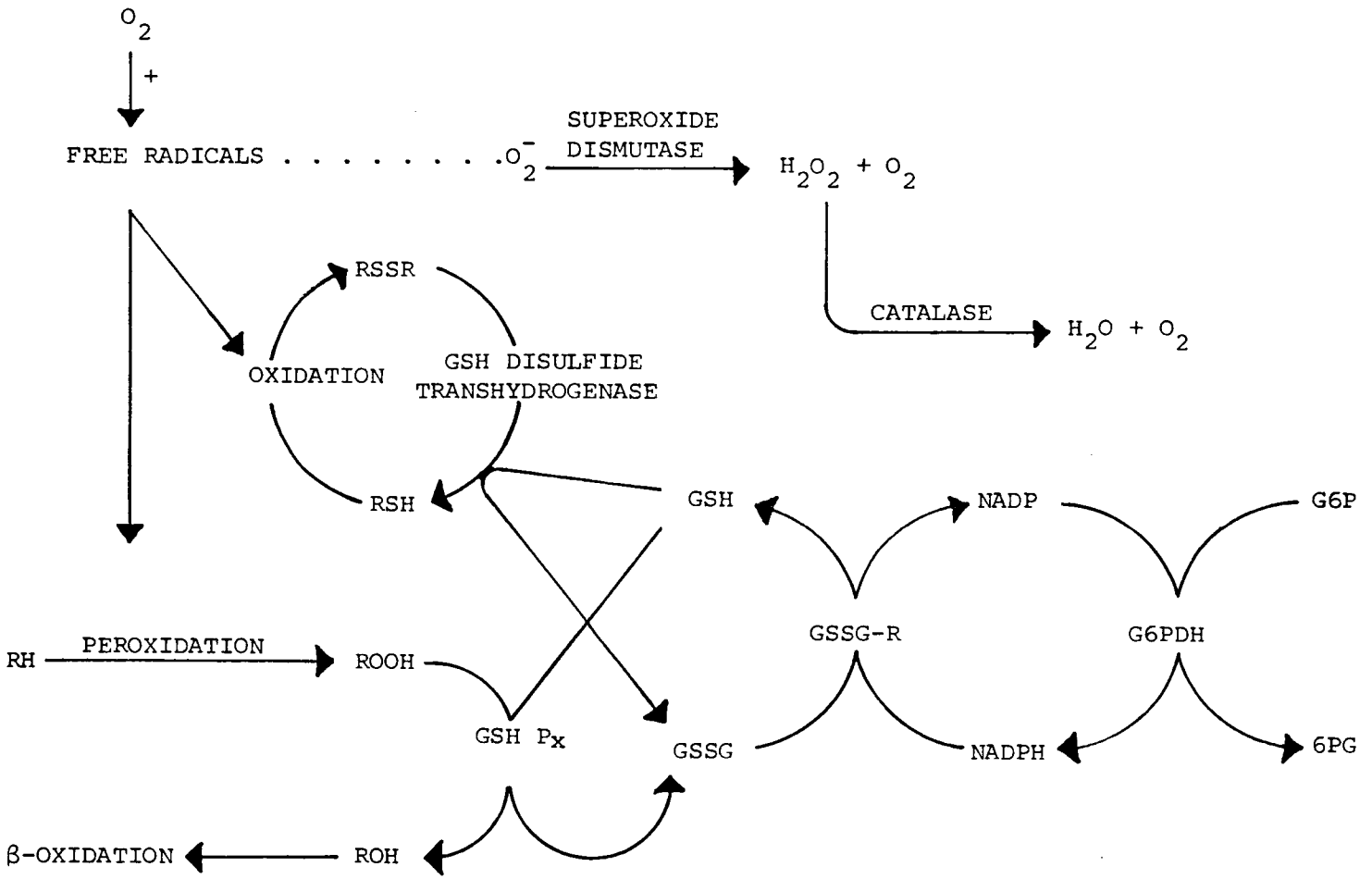


Figure 3. Synergistic mechanism of antioxidant enzymatic action proposed to protect cells from damage mediated by O_2 and lipid peroxides. (Adapted from Kimball et al. (49).)

Glutathione Reductase

Glutathione reductase (NADPH: oxidized glutathione oxidoreductase, E.C. 1.6.4.2) catalyzes the following reaction:

$$GSSG + NADPH + H^+ \xrightarrow{GSSG\text{-}R} 2\ GSH + NADP^+$$

GSSG-R is specific for GSSG (50) and reacts with NADPH or NADH. The reaction with NADH is inhibited by NaCl and KCl and is stimulated by $HPO_4^=$ (51). Rat liver GSSG-R was found to have a molecular weight of 44,000 and to contain one active site per molecule of enzyme (31). The K_m for GSSG is 6.1×10^{-5} M, and for NADPH is 7.6×10^{-6} M (phosphate buffer, pH 7.6; 25°). For the enzyme in human serum, the pH optimum in phosphate buffer and tris-maleate buffer is between 6.4 and 6.7 (52). The enzyme is located primarily in the cytoplasm of the cell (30). Wendell (30) presented evidence that the GSSG-R reaction does not lie near its thermodynamic equilibrium. GSSG-R is inhibited by purine nucleotides and this inhibition is competitive with GSH (53). There is evidence that GSSG-R levels remain relatively constant under a range of perfusion conditions known to produce marked changes in the pattern of energy-producing metabolism (30).

Glutathione Peroxidase

Glutathione peroxidase (glutathione: hydrogen peroxide oxidoreductase, E.C. 1.11.1.9) catalyzes the following reaction:

$$2GSH + H_2O_2 \xrightarrow{GSH\text{-}Px} GSSG + 2\ H_2O \qquad (54)$$

GSH-Px is specific for thiols, especially GSH, as H-donors, but probably accepts any hydro-peroxide as substrate (55). The enzyme was first discovered by Mills (56), who demonstrated that this activity was distinct from catalase. The enzyme is not inhibited by CN^-, N_3^-, F^- or peroxide, and it is probably not a flavoprotein (55). The K_m for GSH in mammalian erythrocytes is about 1.0×10^{-2} M, and for H_2O_2 is about 2.5×10^{-5}M (phosphate buffer, pH 7.0; 20°) (54). The optimum pH for rat liver enzyme was 9.0 (55). GSH-Px from erythrocytes consists of four subunits (84,000 daltons total) with one gram-atom of selenium per subunit (57), and it acts synergistically with vitamin E to protect the cell from peroxidative damage (58). It was demonstrated that GSH-Px activity in rat tissues increases with increased dietary levels of selenium (58). The activities of GSH-Px and GSSG-R increase in rats undergoing oxidative stress (58). The rate of the GSH-Px reaction depends on the availability of substances which, on oxidation by O_2, can form H_2O_2 (25). Thus, the rate-limiting step in GSSG formation appears to be

the production of peroxide substrate rather than the enzyme levels (32).

The enzyme has the highest specific activities in the mitochondrial and supernatant fractions of the cell (32, 55). In the rat, high specific activities were found in liver and in blood, with very little in intestinal mucosa. Rat liver enzyme is similar in properties to the erythrocyte GSH-Px studied by Paglia and Valentine (54, 55).

CHAPTER III

EXPERIMENTAL PROCEDURE

I. BASIC DESIGN

The investigations reported by Bittle[1] showed an effect of dietary sulfate on the controlling enzymes of lipogenesis, but raised a question with respect to the source of the reducing equivalents. The present study was designed, therefore, to determine whether dietary inorganic sulfate had an effect on rat liver glucose-6-phosphate dehydrogenase activity and, consequently, on the production of reducing equivalents needed for lipogenesis. Following a preliminary study demonstrating that dietary sulfate did affect G6PDH activity, additional experiments were conducted to determine the mechanism of this effect. The study was composed of the following three experiments:

Experiment 1. A preliminary determination of the effect of dietary sulfate of G6PDH activity in rat liver cytosol.

Experiment 2. Determination of the effect of dietary sulfate of the activities of GSSG-R and GSH-Px.

Experiment 3. Determination of the in vitro effect of $SO_4^=$ and GSSG on the variable G6PDH activities in the livers

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[1]Bittle, op. cit.

of rats fed diets with variable sulfate levels, and of the tissue glutathione levels to determine if sulfate associated changes in tissue glutathione levels could be involved in the G6PDH effect.

II. DIETS

The composition of the purified diets used in these studies is described in Tables 1 and 2. The diets shown in Tables 1 and 2 and the salt mixture formula are identical to those used by Bittle.[2] Although only seven diets are described in Tables 1 and 2, three groups of diets containing each of the three sulfate levels are actually represented, as shown in Table 3. The three classifications include constant total sulfur-variable methionine diets (CTS-VM), constant total sulfur-variable cysteine diets (CTS-VC), and constant cysteine diets (CC). The levels of organic sulfur (sulfur containing amino acid) as sulfate are equivalent for all 0.02% $SO_4^=$ diets. The diets containing 0.02% $SO_4^=$ are considered control diets, since this level of $SO_4^=$ has previously been established as the optimal level in the rat diet (2). Two pairs of diets listed in Table 3 are identical: (1) the 0.42% $SO_4^=$, CTS-VM diet is identical to the 0.42% $SO_4^=$, CTS-VC diet, and (2) the 0.02% $SO_4^=$, CTS-VC diet is identical to the 0.02% $SO_4^=$ CC diet.

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[2]Bittle, op. cit.

Table 1

Composition of Diets Supplemented with Methionine

Component	0.0002% $SO_4^=$	0.02% $SO_4^=$	0.42% $SO_4^=$
		g/100g	
Casein[a]	15.00	15.00	15.00
Sucrose	30.00	30.00	30.00
Cornstarch	30.00	30.00	30.00
Vegetable Oil[b]	2.00	2.00	2.00
Vegetable Shortening[c]	10.00	10.00	10.00
Vitamin Mixture[d]	2.00	2.00	2.00
Basal Salt Mixture[e]	1.34	1.34	1.34
Non-nutritive Bulk[f]	7.66	7.68	8.00
$CaCO_3$	1.34	1.32	0.92
$CaSO_4 \cdot 2H_2O$	0.00	0.04	0.75
Methionine	0.66	0.62	0.00

[a]Nutritional Biochemicals Corp., Cleveland, Ohio 44128.

[b]Wesson Oil, Hunt-Wesson Foods, Inc., Fullerton, CA 92634.

[c]Crisco, Proctor and Gamble, Cincinnati, Ohio 45202.

[d]Nutritional Biochemicals Corp., 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, inositol 510, choline chloride 75, menadione 2.25, biotin 0.020, folic acid 0.090, vitamin B_{12} 0.00135, α-tocopherol 5.0, vitamin A 9×10^5 units, vitamin D 1×10^5 units, and enough glucose to make 1000 g.

[e]Formulated to supply the following amounts of minerals (g/450 g salt mixture): magnesium carbonate 30.6, sodium chloride 69.0, potassium chloride 112, potassium monobasic phosphate 212, ferric phosphate 20.5, potassium iodide 0.08, sodium fluoride 0.1, manganese chloride 0.4, aluminum potassium sulfate 0.1, and cupric acetate 0.72.

[f]Alphacel, Nutritional Biochemical Corp., Cleveland, Ohio 44128.

Table 2

Composition of Diets Supplemented with Cysteine

Component	0.0002% $SO_4^=$	0.0002% $SO_4^=$	0.02% $SO_4^=$	0.42% $SO_4^=$
			g/100g	
Casein[a]	15.00	15.00	15.00	15.00
Sucrose	30.00	30.00	30.00	30.00
Cornstarch	30.00	30.00	30.00	30.00
Vegetable Oil[b]	2.00	2.00	2.00	2.00
Vegetable Shortening[c]	10.00	10.00	10.00	10.00
Vitamin Mixture[d]	2.00	2.00	2.00	2.00
Basal Salt Mixture[e]	1.34	1.34	1.34	1.34
Non-nutritive Bulk[f]	7.82	7.79	7.80	7.49
$CaCO_3$	1.34	1.34	1.32	0.92
$CaSO_4 \cdot 2H_2O$	0.00	0.00	0.04	0.75
Cysteine	0.530	0.505	0.505	0.505

[a]Nutritional Biochemicals Corp., Cleveland, Ohio 44128.

[b]Wesson Oil, Hunt-Wesson Foods, Inc., Fullerton, CA 92634.

[c]Crisco, Proctor and Gamble, Cincinnati, Ohio 45202.

[d]Nutritional Biochemicals Corp., 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, inositol 510, choline chloride 75, menadione 2.25, biotin 0.020, folic acid 0.090, vitamin B_{12} 0.00135, α-tocopherol 5.0, vitamin A 9×10^5 units, vitamin D 1×10^5 units, and enough glucose to make 1000 g.

[e]Formulated to supply the following amounts of minerals (g/450 g mixture): magnesium carbonate 30.6, sodium chloride 69.0, potassium chloride 112, potassium monobasic phosphate 212, ferric phosphate 20.5, potassium iodide 0.08, sodium fluoride 0.1, manganese chloride 0.4, aluminum potassium sulfate 0.1, and cupric acetate 0.72.

[f]Alphacel, Nutritional Biochemical Corp., Cleveland, Ohio 44128.

Table 3

Classification of Semi-Purified Diets Including Levels of Inorganic and Total Sulfur as Sulfate and of Sulfur Containing Amino Acids

Classification	Inorganic $SO_4^=$	Sulfur Containing Amino Acids	Total Sulfur as $SO_4^=$
		% of Diet	
Constant Total Sulfur-Variable Methionine (CTS-VM)	0.0002	0.66	0.67
	0.02	0.62	0.67
	0.42	0.00	0.67
Constant Total Sulfur-Variable Cysteine (CTS-VC)	0.0002	0.530	0.67
	0.02	0.505	0.67
	0.42	0.000	0.67
Constant Cysteine (CC)	0.0002	0.505	0.65
	0.02	0.505	0.67
	0.42	0.505	1.07

All rats were fed a nonpurified[3] and tap water ad libitum from weaning until they weighed approximately 350 to 450 g for experiments 1 and 2, and until they weighed 125 to 225 g for experiment 3. At that time, they were transferred to purified diets.

Rats in experiment 1 were fed the CTS-VM diets and the CC diets. Rats in experiment 2 were fed only the CTS-VM diets. All three groups of diets were used in experiment 3. The semi-purified diets were fed for periods of 17 to 22 days (experiments 1 and 2), to provide a margin of safety, since metabolic alterations associated with the dietary level of inorganic sulfate have been detected within a period of 14 days (6). In experiment 3, rats were fed the cysteine supplemented diets for periods of 26 to 29 days and the CTS-VM diets for periods of 34 to 41 days. Each test group contained five to six rats per diet.

III. EXPERIMENTAL ANIMALS

Randomly selected adult male rats of a Wistar, Sprague-Dawley, Long-Evans mixed strain served as tissue donors. The animals were housed in galvanized steel cages, with no more than four animals per cage. On days that enzyme assays were

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[3]Rat Chow, formula No. 5012, Ralston Purina Company, Saint Louis, Missouri 63188. Approximate inorganic sulfate composition equals 0.02%.

conducted, one or two rats from each experimental diet group were stunned, decapitated and exsanguinated between 8:00 and 9:00 a.m. Livers were rapidly excised, placed in 100 ml tared beakers containing approximately 50 ml of isotonic KCl (0.154 M) and imbedded in crushed ice, and weighed by difference.

IV. PREPARATION OF LIVER HOMOGENATES

Immediately following excision of livers, 20% homogenates were prepared with cold isotonic KCl (experiments 1 and 3) or cold phosphate buffer (0.1M, pH 7.4) containing 1 mM ethylene-diaminetetraaceticacid (EDTA) (experiment 2). Homogenates were prepared using a motor driven homogenizer[4] with a teflon pestle. The homogenates were then centrifuged at 8,000 × g in a refrigerated centrifuge[5] at 0° for 10 minutes to sediment the nuclei and the mitochondria. This preparation was used for assaying GSSG-R activity, since it was found to have a higher activity than that of the supernatant fraction. The supernatant fraction of the homogenate used in all other enzyme assays was prepared by recentrifuging the mitochondria-free preparation at approximately 100,000 × g for 40 minutes

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[4]Thomas Size C Homogenizer, Thomas Co., Philadelphia, PA.

[5]Lourdes Beta-fuge, Model A, Lourdes Instrument Corporation, 656 Montauk Ave., Brooklyn 8, N.Y.

at 0° in a preparative ultracentrifuge.[6] The mitochondria-free preparations and the supernatant fractions were decanted into vials immediately following centrifugation and kept on ice until used. All enzyme assays and reduced glutathione assays were done on the day of sacrificing.

V. METHODS

Enzyme Assays

Determination of Glucose-6-Phosphate Dehydrogenase Activity

G6PDH activities of rat liver supernatant fractions were determined by a modification of the method of Bottomley et al. (59). The basis of this method is the spectrophotometric measurement of the rate of NADPH formation from the increase in absorbance at 340 nm (14).

The reaction mixture contained the constituents listed in Table 4 in a final volume of 3 ml, including 0.1 ml of supernatant. Blanks consisted of all of the reagents except G6P. In experiment 1, all reagents were mixed on the day of the test, and in experiment 3, in order to minimize variability between days, reagents were mixed in a single quantity at the beginning of the experiment and frozen in batches which

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[6]Beckman preparative ultracentrifuge Model L550, Serial No. 50328, rotor type 50, #2778, Beckman Instruments, Inc., Scientific Instruments Division, Campus Dr. at Jamboree Blvd., Ervine, CA 92713.

Table 4

Components of the Reaction Mixture Used to Determine Glucose-6-Phosphate Dehydrogenase Activity

Component	Concentration
	µmoles/tube
KCl	138.6
Tris-HCl buffer, pH 8.0	360.0
$MgCl_2$	30.8
Nicotinamide	30.0
Glucose-6-phosphate[a]	6.0
NADP[a]	1.0

[a]Sigma Chemical Co., P. O. Box 14508, St. Louis, MO 63178.

were thawed at 4° overnight immediately preceding the day of testing. Exactly 2.9 ml of the cold reaction mixture was added to individual test tubes and allowed to equilibrate with the temperature of the room in which measurements were made (approximately 18°). The reaction was initiated by adding the supernatant fluid to blank and sample tubes. The contents were poured into silica cuvettes and mixed by pouring back into the original tube, then back into the silica cuvette. Then the optical density (O.D.) of the sample was measured each minute against the blank at 340 nm from 1 to 3 minutes (experiment 1), or from 4 to 8 minutes (experiment 3), with a Beckman DU spectrophotometer.[7] Samples were run in triplicate in experiment 1; however, as differences between triplicates were small, single samples were run in experiment 3.

The enzyme activity of G6PDH was expressed as μmoles NADPH produced per minute/gram of protein as calculated from the extinction coefficient (E^{mM}_{340}) of NADPH (27) and the slope of the line when O.D. was plotted versus time yielding the change in O.D. per minute (ΔE/min), as shown in the following formula:

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[7]Beckman Quartz Spectrophotometer, Model DU, Serial No. 97238 (Beckman Instruments, Inc., Scientific Instruments Division, Irvine, CA 92713) modified to solid state configuration by Update Instrument, Inc. (Model 11C, Serial No. 2005), P. O. Box 5177, Madison, Wisconsin 53705.

μmoles NADPH produced per min/g protein =

$$\frac{(\Delta E/\text{min}) \times 3 \text{ ml}}{6.2(E_{340}^{mM}) \times (\text{g protein}/0.1 \text{ ml supernatant fluid})}$$

In experiment 3, the effects of oxidized glutathione and sulfate on G6PDH activity and on the differences in the enzyme activities among the dietary groups were assessed. This was done by adding 5 μg $SO_4^=$ or 50 μg GSSG to sample tubes. These were run simultaneously with the previously mentioned samples. The level of GSSG used was about half the mM concentration in rat liver as estimated by Wendell (30), as measurements of GSSG levels are thought to be over-estimates (25) based on the rapid oxidation of GSH. The level of $SO_4^=$ used was previously determined to be within physiological ranges (see Results section).

Determination of Glutathione Reductase Activity

The GSSG-R activities of the mitochondria free preparations in experiment 2 were determined by a modification of the method described by Horn (52) for serum. Addition of mercaptoethanol to the reaction medium was necessary with this liver preparation to prevent the reoxidation of GSH as it was formed, as the basis of this method is the spectrophotometric measurement of the disappearance of NADPH as it reacts with GSSG to form GSH and NADP (see discussion of

GSSG-R in Chapter II). Levels of mitochondria free homogenate, GSSG and mercaptoethanol were varied in preliminary tests in order to determine the optimum levels of each. The reaction mixture contained the constituents listed in Table 5 in a final volume of 3 ml including 0.4 ml of mitochondria free homogenate diluted 10-fold. The given concentration of mercaptoethanol is equivalent to approximately 0.05 ml of a 100-fold dilution. The reaction was run at pH 7.4, in order to mimic physiological conditions and so that GSSG-R and GSH-Px activities could be compared at the same pH. Blanks contained all of the reagents except GSSG. After adding cold reagents to each tube, the reaction mixtures were allowed to equilibrate with the room temperature (approximately 18°). The reaction was initiated by adding the mitochondria free homogenate to the sample and blank tubes. Tube contents were then poured into silica cuvettes, mixed as in the G6PDH assay, and the optical densities of the samples were measured each minute against the blanks (set at 0.8 O.D. units) at 340 nm from 1 to 3 minutes with a Beckman DU spectrophotometer. The change in O.D. per minute (ΔE/min), as determined by the difference between the 1 minute and 3 minute measurements divided by two, was used to calculate the µmoles of NADPH disappearing per minute/gram protein by the following formula:

Table 5

Components of the Reaction Mixture Used to Determine Glutathione Reductase Activity

Component	Concentration
	µmoles/tube
Phosphate buffer, pH 7.4	195.0
GSSG[a]	3.0
NADPH[a]	1.2
Mercaptoethanol	0.2

[a]Sigma Chemical Company, P. O. Box 14508, St. Louis, MO 63178.

μmoles NADPH disappearing per min/g protein =

$$\frac{(\Delta E/\text{min}) \times 3 \text{ ml}}{6.2\ (E^{mM}_{340}) \times (\text{g protein } 0.04 \text{ ml homogenate})}$$

Determination of Glutathione Peroxidase Activity

The activity of GSH-Px in the rat liver supernatant fractions was determined by a modification of the method of Pagalia and Valentine (54), used to determine the activity of GSH-Px in erythrocytes. A similar method was used by Little and O'Brien (55) on a partially purified sample of the enzyme from perfused rat liver supernatant fluid. The basis of this test is the spectrophotometric measurement of NADPH disappearance as it reacts with GSSG in the presence of an excess of GSSG-R. NADPH disappearance thus becomes a measure of the amount of GSSG produced by the GSH-Px catalyzed reaction and, consequently, a measure of GSH-Px activity as seen in the following reaction:

$$2\ GSH + H_2O_2 \xrightarrow{GSH\text{-}Px} 2\ H_2O + GSSG \xrightarrow[NADPH]{GSSG\text{-}R} \underset{NADP}{2\ GSH}$$

Levels of supernatant, GSH and H_2O_2 were varied in preliminary tests in order to determine the levels needed for maximal activity. The reaction mixture contained the constituents listed in Table 6 in a final volume of 3 ml, including 0.1 ml of the supernatant preparation diluted 100-fold. Blanks

Table 6

Components of the Reaction Mixture Used to Determine Glutathione Peroxidase Activity

Components	Concentration
	µmoles/tube
Phosphate buffer, pH 7.4	125.0
EDTA	1.0
NADPH[a]	0.84
GSH[a]	30.0
H_2O_2	0.2
GSSG-R[a] (1.0 e.u.)	

[a]Sigma Chemical Company, P. O. Box 14508, St. Louis, MO 63178.

contained all of the reagents except GSH. Nonenzymatic oxidation of GSH was determined by simultaneous assay of a similar reaction mixture, excluding the supernatant fluid. The reaction was initiated by addition of the H_2O_2 to the sample, blank and nonenzymatic assay tubes. The contents were transferred to silica cuvettes, mixed, and the O.D. of the sample and nonenzymatic assay systems was measured against the blank (set at 0.8 O.D. units) at 340 nm from 1 to 3 minutes with a Beckman DU spectrophotometer (room temperature, 18°). The reaction rate (ΔE/min, as determined by the difference between the 1 minute and 3 minute measurements divided by two) of the nonenzymatic system was subtracted from the reaction rate of the sample system to determine the true enzymatic activity according to the following formula:

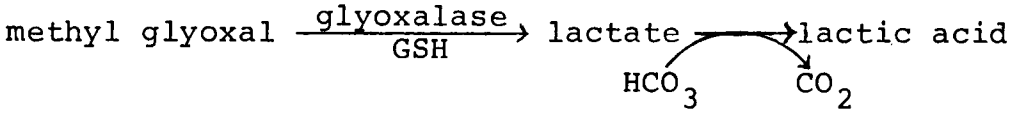
$$\mu\text{moles NADPH disappearing per min/g protein} = \frac{(\Delta E_{enz}/\text{min} - \Delta E_{nonenz}/\text{min}) \times 3 \text{ ml}}{6.22\ (E_{340}^{mM}) \times (\text{g protein}/1.0 \times 10^{-3}\text{ml supernatant fluid})}$$

Determination of Reduced, Oxidized and Total Glutathione Levels

Reduced and total glutathione levels were determined by the method of Settle.[8] This is a manometric method, the

[8]Settle, E. A. (1977) An investigation of the relationship between sulfur nutritional status and lung

basis of which is the measurement of CO_2 liberated from a bicarbonate medium by lactic acid which is formed from methyl glyoxal in the presence of glyoxalase and catalytic amounts of GSH according to the following formula:



Immediately following centrifugation, 2 ml of the mitochondria free homogenate (experiment 2) or the supernatant fluid (experiments 2 and 3) was mixed, using a vortex mixer, with an equal volume of cold 2% sulfosalicylic acid. These mixtures were centrifuged at about 700 × g for about 10 minutes in order to remove the protein precipitate. These preparations were kept in an ice bath until used.

The glyoxylase enzyme was obtained from baker's yeast, as described by Settle.[9] Approximately 180 g (12 cakes) of fresh pressed baker's yeast were crumbled, weighed and mixed by stirring with 720 ml of cold (4°) acetone for 10 minutes. The acetone was then removed rapidly by suction filtration, but the yeast was not allowed to dry completely. The above procedure was repeated by extracting the residue with an additional 240 ml of cold acetone.

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neoplasms induced by benzo(a)pyrene in rats. Unpublished doctoral dissertation, The University of Tennessee, Knoxville.

[9]Ibid.

To remove the glutathione from the glyoxylase preparation, the mixture was washed with cold distilled water. The residue was suspended and stirred for 10 minutes with 288 ml cold distilled water. The solid enzyme-containing portion was then collected by centrifuging at 4100 × g in a refrigerated centrifuge for about 10 minutes and decanting the water. This washing process was repeated five times. Finally, the residue was dried with acetone and ether in the following manner. The residue was dried by suspending it in 240 ml of cold acetone and blended in a Waring blender. The acetone was then removed by suction filtration. This acetone wash was repeated twice, then, immediately following the final filtration, 144 ml of cold ether was poured over the residue in the suction filter. The residue was then immediately spread on paper and quickly worked with the hands until dry. The glyoxylase preparation was then weighed and stored at -20° in a tinted glass jar until used.

The measurement of total and reduced glutathione was carried out in a Warburg apparatus preheated to 25°. The reaction mixtures were prepared in an ice bath by mixing the components shown in Table 7. A 1.5 ml portion of each reaction mixture was pipetted into the main compartment of Warburg flasks. An 0.5 ml portion of the mitochondria-free (experiment 2) or supernatant (experiments 2 and 3) preparations with sulfosalicylic acid was pipetted into the side

Table 7

Components of the Reaction Mixture Used to Determine Total and Reduced Glutathione

Component	Amount Added for Total GS Reaction Mixture	Amount Added for GSH Reaction Mixture
	ml	ml
Glyoxalase Preparation (5 g)		
Distilled Water	19.0	19.25
$NaHCO_3$ (0.2 M)	14.90	14.90
Methyl Glyoxal (40%)	0.75	0.75
2-Mercaptoethanol	0.25	0.00

arms. A blank was prepared by pipetting 0.5 ml of a 1:1 mixture of phosphate buffer (experiment 2) or KCl (experiment 3) and 1% sulfosalicylic acid to the side arm of a flask containing the reaction mixture. A standard solution containing 5 mg GS per 100 ml of this 1:1 mixture was also run by adding 0.5 ml to the side arm.

After the flasks were connected to the manometers, they were placed in the water bath and allowed to equilibrate by shaking for 5 minutes with the system open. Before the contents of the flasks were mixed, the Brodie's solution in the manometers was adjusted to read 150 mm on the right hand scale. The contents of the flasks were mixed and then, following an additional 4 minutes, the systems were closed. Zero-time readings were taken 5 minutes after the systems were closed by adjusting the right hand scales to 150 mm and reading the left hand scales.

The rates of change (the change in mm Brodie's solution per minute) were corrected for differences in volume among the systems by multiplying the mm change by the flask (k_f) and manometer (k_m) constants. The flask and manometer constants had been determined for the equipment by the method of Umbreit et al. (60). The corrected rate of change of the blank (h_o) was then subtracted from the sample (h_s) and standard (h_{std}) rates. Concentrations of total glutathione (GS) and GSH were calculated by comparing the rates of change

of the samples to the rates of change of the standard, according to the following formula:

mg GS or GSH/g protein =

$$\frac{\dfrac{(h_s k_f k_m) - h_o k_f k_m)}{(h_{std} k_f k_m) - (h_o k_f k_m)} \times 5 \text{ mg GS/100 ml} \times 2}{\text{g protein per ml supernatant fluid or homogenate}}$$

GSSG concentrations were obtained by subtraction of GSH concentrations from GS concentrations.

After the readings were completed, the systems were opened, the Brodie's solution in the manometers was lowered and the flasks were removed from the manometers. The flasks were degreased with acetone and hot water and then cleaned by the permanganate method of Ryan.[10] The degreased flasks were washed in distilled water and soaked for 30 minutes in a 2% potassium permanganate solution made with 50% sodium hydroxide. The flasks were then rinsed in distilled water and soaked in a saturated oxalic acid solution until bleached. The flasks were rinsed again in distilled water, placed in a pyrex dish, covered with distilled water and boiled on a hot plate for about 15 minutes, then placed in a convection oven to dry.

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[10]Ryan, W. L. (1953) A metabolic study of cold shock in mammalian spermatozoa. Unpublished doctoral dissertation, The University of Missouri, Columbia.

Determination of Tissue Sulfate Levels

In order to determine the physiological level of sulfate to be used for determining the effect of sulfate on G6PDH activity, it was necessary to first develop an accurate method for this determination. A simple barium precipitation method for free $SO_4^=$ would be subject to error in the presence of large amounts of protein, since protein interferes with the binding of barium to sulfate and prior precipitation of the protein results in the absorption of part of the free sulfate by the protein. A radioassay for $SO_4^=$ was developed, with reference to Tonzatich (61), to eliminate these sources of error. The basis of this method is the measurement of specific activity (CPM/μg $SO_4^=$) and the concentration (μg $SO_4^=$/ml) of solutions in which the supernatant fluid and $^{35}SO_4^=$ are mixed prior to protein precipitation. Following protein precipitation, the $SO_4^=$ is precipitated with Ba^{++} so that the competition for Ba^{++} between the radioactive and nonradioactive $SO_4^=$ is not affected by the removal of the protein, which is accompanied by a decrease in total free $SO_4^=$.

It was necessary to determine what level of each reagent was needed to obtain a clear competition between the radioactive $SO_4^=$ and the $SO_4^=$ in the supernatant fluid. This was accomplished by varying the levels of supernatant fluid,

$^{35}SO_4^=$, Na_2SO_4 (standards) and $BaCl_2$, until a clear and consistent competition between the $^{35}SO_4^=$ and the $SO_4^=$ in the supernatant fluid was observed, as evidenced by a decrease in the specific activity. Supernatant levels were varied from 0.3 ml to 5.0 ml/tube. Radioactive sulfate levels were varied from 0.005 µmoles to about 100 µmoles/tube. Sodium sulfate standards were varied from 1 ppm (0.011 µmoles) to 100,000 ppm (1100 µmoles/tube). $BaCl_2$ was varied from 5 to 50 µmoles/tube in an initial test in which excess sulfate was used. This method was not feasible because the levels of sulfate in the samples were so low that the $BaSO_4$ was not precipitating equally in all samples. This method was based on the assumption that all of the Ba^{++} was precipitated, thus $SO_4^=$ levels were constant, and no Ba^{++} assay was done. Following this observation, an excess of $BaCl_2$ was used and Ba^{++} determinations were run as a measure of precipitated $SO_4^=$.

Competitive binding between the sulfate in the supernatant and the $^{35}SO_4^=$ was observed in the following procedure. A radioactive standard was prepared to contain 0.5 µg $^{35}SO_4^=$,[11] and approximately 3.3×10^6 CPM (1.5 µC) per ml. A nonradioactive Na_2SO_4 standard containing 2000

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[11] Sulfur-35 as sulfate, carrier free, pH 6-8, Amersham Corp., 2636 South Clearbrook Drive, Arlington Heights, IL 60005.

ppm $SO_4^=$, a 15% (w/v/) $BaCl_2$ solution, a 1.0 N HCl solution, an 0.5% (w/v) disodium EDTA solution made with 1.0% (w/v) NaOH and a 10% (w/v) trichloracetic acid (TCA) solution were also prepared. Using an automatic pipette with disposable tips, in order to avoid contamination with $^{35}SO_4^=$, 1.0 ml of the $^{35}SO_4^=$ standard was added to each of six test tubes. Then 0.250 (0.5 µg $SO_4^=$) and 0.500 (1.0 µg $SO_4^=$) ml portions of the Na_2SO_4 standard solution were added to two of these tubes and 5.0 ml of supernatant was added to each of two others. Two tubes, containing only the radioactive standard, were run as zero $SO_4^=$ standards. Enough water was added to each tube to make a final volume of 6.0 ml. The tube contents were mixed with a vortex mixer and allowed to stand about 30 minutes. Then 1.0 ml of 10% TCA was added to each tube to precipitate the protein and the tube contents were mixed with a vortex mixer and centrifuged at about 700 × g for 10 minutes. The contents of the tubes were decanted into separate test tubes and the precipitates were washed twice with 1.0 ml of 10% TCA by mixing, centrifuging and decanting the liquid into the respective tubes, discarding the precipitate. This was done to collect any remaining $SO_4^=$. An excess of $BaCl_2$ (1 ml of 15% $BaCl_2$) was then added to each tube containing the decanted liquid. The tube contents were mixed with a vortex mixer and allowed to stand for about 1 hour. The tubes were then centrifuged at 700 × g for about

20 minutes and the liquid discarded. The precipitate was washed twice with 1.0 N HCl by mixing 3 ml of the HCl solution with the precipitate then recentrifuging as above and pouring off the liquid to remove any remaining $BaCl_2$. HCl was used to increase the solubility of the $BaCl_2$ (62). Next, the $BaSO_4$ precipitate was dissolved in the EDTA-NaOH solution, brought to a final volume of 10 ml using volumetric flasks. Complete dissolution of the $BaSO_4^=$ was accomplished by allowing the flasks to stand for about 1 hour, intermittently inverting the flasks. The Ba^{++} content of these solutions was determined by atomic absorption spectrometry,[12] according to a modification of the method of Roe et al. (63). By comparing these values to a standard containing 1.0 µg of $SO_4^=$ treated with $BaCl_2$, the $SO_4^=$ concentrations of these solutions were calculated. The radioactive sulfate present in the solutions was determined by transferring 0.3 ml of each solution to glass vials containing 6 ml of ethoxyethanol then washing the tubes with 10 ml of PPO in toluene and transferring these washings to the vials. The vials were then placed in a scintillation counter[13] and the solutions

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[12]Atomic Absorption Spectrometer, Perkin-Elmer Model 303, Norwalk, Conn.

[13]Beckman Liquid Scintillation System, Model LS100C, Serial No. 1000923, Beckman Instruments, Inc., Scientific Instruments Division, Ervine, CA 92713.

were counted for 1 minute on the ^{14}C channel, since ^{14}C has a similar decay energy to that of ^{35}S (64). Specific activities of the solutions were calculated by dividing the CPM by the $SO_4^=$ concentrations. The sulfate concentration in the supernatant samples was calculated by the following formula:

$$\mu g\ SO_4^=/ml\ \text{supernatant fluid (s. f.)} =$$

$$\frac{0.5\ \mu g\ SO_4^=}{5.0\ ml\ s.\ f.} \times \frac{(CPM/\mu g\ SO_4^=)_A - (CPM/\mu g\ SO_4^=)_S}{(CPM/\mu g\ SO_4^=)_A - (CPM/\mu g\ SO_4^=)_B}$$

where $(CPM/\mu g\ SO_4^=)_A$ represents the specific activity of the zero sulfate standard, $(CPM/\mu g\ SO_4^=)_B$ represents the specific activity of the 0.5 µg/tube standard and $(CPM/\mu g\ SO_4^=)_S$ represents the specific activity of the sample.

Determination of Tissue Protein Levels

The protein content of the mitochondria free homogenates and supernatant fractions was determined by the method of Lowry et al. (65). Solutions of 2% Na_2SO_4 in 0.1N NaOH, 0.5% $CuSO_4 \cdot 5H_2O$ and 1% NaK-tartrate were prepared and Folin-Ciocalteu phenol reagent was diluted about two-fold (to make a 1 N solution based on a titration with 1.0 N NaOH to a phenolphthalein end-point). The days on which assays were run, a mixture was made containing 100 ml of the 2% Na_2CO_3 solution, 1.0 ml of the $CuSO_4$ solution and 1.0 ml of the

NaK-tartrate solution. A protein standard containing 0.02g bovine serum albumin per 100 ml of 0.5 N NaOH was also prepared. The homogenate or supernatant was diluted 46 fold (0.1 ml homogenate or supernatant per 4.5 ml 0.5 N NaOH) or 51 fold (0.1 ml homogenate or supernatant per 5.0 ml 0.5 N NaOH). To a tube containing 1.0 ml of the diluted homogenate or supernatant, or 1.0 ml of the standard, was added 5.0 ml of the Na_2CO_3-$CuSO_4$ KaK-tartrate solution. The contents of the tubes were mixed with a vortex mixer and allowed to stand for at least 10 minutes at room temperature. Then 0.5 ml of dilute Folin reagent was added very rapidly to each tube and mixed within a second or two using the vortex mixer. After 30 minutes, the optical densities of the samples were measured in a spectrophotometer[14] at 750 nm. The protein concentrations of the samples were calculated by the following formula:

mg protein/ml homogenate or supernatant fluid =

$$\frac{\text{O.D. sample}}{\text{O.D. standard}} \times 0.2 \text{ mg protein/ml} \times \text{dilution factor}$$

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[14]Beckman B spectrophotometer, Serial No. 012000-1000295, Beckman Instruments, Inc., Scientific Instruments Division, Campus Dr. at Jamboree Blvd., Ervine, CA 92713.

Statistics

Differences between sulfate levels were analyzed by either the t-test for paired comparisons or by the t-test for unequal sample sizes, as described by Lewis (66). Differences were considered significant if t values were greater than the tabulated t value for a 90% confidence interval ($P < 0.1$).

Widely variable data were eliminated by the Q-test, described by Day and Underwood (67). This involved comparing the ratio of the difference between the suspect result and its nearest neighbor, and the range of the results to a table of Q values. If the computed Q value was greater than the value in the table for a given sample size and a 90% confidence interval, the suspect result was discarded.

CHAPTER IV

RESULTS

I. EXPERIMENT 1

The purpose of Experiment 1 was to determine the effect of dietary sulfate on rat liver G6PDH activity. The CTS-VM and CC diets with varying sulfate levels were used. When total dietary sulfur was kept constant by varying methionine levels, G6PDH activity was found to be significantly higher ($P < 0.1$) at the low sulfate level than when inorganic sulfate was fed at the established optimum of 0.02% (Table 8). The higher level of sulfate did not produce activities significantly different from the optimum level. This test was repeated and similar results were obtained so the data were pooled. When dietary cysteine was kept constant, no differences were found in G6PDH activity among the different sulfate levels (Table 9).

II. EXPERIMENT 2

In Experiment 1, an effect of dietary sulfate on G6PDH activity was observed when total dietary sulfur was kept constant by varying the level of methionine. As a controlling effect of oxidized glutathione on G6PDH activity has been proposed (24, 25) and since glutathione peroxidase and

Table 8

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Activity of Rat Liver Glucose-6-Phosphate Dehydrogenase when the Total Sulfur Was Kept Constant by Varying the Level of Methionine

% Dietary Sulfate	% Dietary Methionine	Glucose-6-Phosphate Dehydrogenase Activity[a]	
		μmoles NADPH/min[b] g protein	
0.0002	0.66	20 ± 7*[c]	(12)[d]
0.02	0.62	14 ± 4*	(10)
0.42	0.00	16 ± 5	(12)

[a]Reported values are pooled data from two studies.

[b]Values are means ± SD.

[c]All means followed by asterisk are significantly different ($P < 0.05$) by the t-test for unequal sample sizes.

[d]Numbers in parentheses indicate the number of animals used.

Table 9

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Activity of Rat Liver Glucose-6-Phosphate Dehydrogenase When the Dietary Cysteine Was Kept Constant

% Dietary Sulfate	% Dietary Cysteine	Glucose-6-Phosphate Dehydrogenase Activity	
		μmoles NADPH/min[a] g protein	
0.0002	0.505	14 ± 1[b]	(5)[c]
0.02	0.505	13 ± 1	(5)
0.42	0.505	12 ± 2	(6)

[a]Values are means ± SEM.

[b]All means followed by asterisk are significantly different ($P < .1$) by the t-test for unequal sample sizes.

[c]Numbers in parentheses indicate the number of animals used.

glutathione reductase play major roles in regulating GSSG levels (24, 25), an experiment was designed to determine the effect of dietary sulfate on GSSG-R and GSH-Px activities when total sulfur was kept constant with methionine supplementation. These measurements were made in an attempt to determine the mechanism of the sulfate effect on G6PDH.

No significant differences ($P < 0.1$) were observed in GSSG-R activity between the diets; however, the activity of GSSG-R did appear lower in the livers of rats fed diets low in sulfate, as shown in Table 10.

Glutathione peroxidase activity was higher in the livers of rats fed the diet containing 0.02% of $SO_4^=$ than of those fed the low or high $SO_4^=$ levels (Table 11), although this difference was significant only between the diets low and optimal with respect to sulfate ($P < 0.1$). Again, a large variability or small sample size might account for the failure to show a significant difference between the optimum sulfate and the high sulfate diets.

III. EXPERIMENT 3

Since differences in GSSG-R and GSH-Px were observed in Experiment 2, an experiment was designed to determine whether there was a corresponding change in tissue GSSG levels and to determine whether changes in GSSG levels could explain the changes in G6PDH activity resulting from the variable levels

Table 10

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Activity of Rat Liver Glutathione Reductase when the Total Sulfur Was Kept Constant by Varying the Level of Methionine

% Dietary Sulfate	% Dietary Methionine	Glutathione Reductase Activity	
		μmoles NADPH/min[a] g protein	
0.0002	0.66	88 ± 5[b]	(5)[c]
0.02	0.62	97 ± 18	(5)
0.42	0.00	99 ± 22	(5)

[a]Values are means ± SD.

[b]All means followed by asterisk are significantly different ($P < 0.1$) by the t-test for paired comparisons.

[c]Numbers in parentheses indicate the number of animals used.

Table 11

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Activity of Rat Liver Glutathione Peroxidase when the Total Sulfur Was Kept Constant by Varying the Level of Methionine

% Dietary Sulfate	% Dietary Methionine	Glutathione Peroxidase Activity	
		μmoles NADPH/min[a] g protein	
0.0002	0.66	770 ± 56*[b]	(3)[c]
0.02	0.62	870 ± 86*	(3)
0.42	0.00	700 ± 270	(3)

[a]Values are means ± SD.

[b]All means followed by asterisk are significantly different ($P < 0.1$) by the t-test for paired comparisons.

[c]Numbers in parentheses indicate the number of animals used.

of dietary sulfate. Thus it was necessary not only to show that changes in GSSG levels corresponded to changes in G6PDH activities, but also that physiological levels of GSSG vitro could eliminate the differential effect of dietary sulfate on G6PDH activity. Also, an in vitro test to determine whether sulfate itself could eliminate the differential effect of dietary sulfate on G6PDH activity was run. The CTS-VM, CTS-VC and CC diets were used in this experiment.

The Effect of Dietary Sulfate on GSSG Levels

When total dietary sulfur was kept constant by varying the levels of methionine, the tissue levels of GSSG decreased significantly ($P < 0.05$) with increasing dietary sulfate (Table 12). This seemed to correspond to decreasing total glutathione levels with increasing dietary sulfate, while GSH levels did not decrease with increasing dietary sulfate levels above the optimum level.

When total dietary sulfur was kept constant by varying the cysteine levels, the tissue GSSG levels decreased significantly ($P < 0.1$) at the sulfate level above the optimum level, but were not affected by low dietary sulfate (Table 13). Again, total glutathione levels decreased with increasing dietary sulfate. In this case, GSH levels also decreased with increasing dietary sulfate but not enough to prevent a significant drop in GSSG levels, as the dietary sulfate level was increased above the optimum level.

Table 12

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Levels of Total, Reduced and Oxidized Glutathione in Rat Liver when the Total Dietary Sulfur Was Kept Constant by Varying the Level of Methionine

% Dietary Sulfate	Total Glutathione	Reduced Glutathione	Oxidized Glutathione	
		mg/g protein[a]		
0.0002	55 ± 39[b]	38 ± 29	18 ± 12*[b]	(5)[c]
0.02	47 ± 32	32 ± 23	15 ± 11*	(5)
0.42	40 ± 31	32 ± 21	9 ± 10*	(5)

[a]Values are means ± SD.

[b]All means followed by asterisk are significantly different ($P < 0.05$) by the t-test for paired comparisons.

[c]Numbers in parentheses indicate the number of animals used.

Table 13

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Levels of Total, Reduced and Oxidized Glutathione in Rat Liver when the Total Dietary Sulfur Was Kept Constant by Varying the Level of Cysteine

% Dietary Sulfate	Total Glutathione	Reduced Glutathione	Oxidized Glutathione	
		mg/g protein[a]		
0.0002	56 ± 43[b]	41 ± 34	15 ± 11	(5)[c]
0.02	53 ± 40	38 ± 28	15 ± 13*	(5)
0.42	40 ± 31	32 ± 21	9 ± 10*	(5)

[a]Values are means ± SD.

[b]All means followed by asterisk are significantly different ($P < 0.05$) by the t-test for paired comparisons.

[c]Numbers in parentheses indicate the number of animals used.

When dietary cysteine was kept constant, the levels of GSSG remained relatively constant and the decreases in total and reduced glutatuione with increasing dietary sulfate were small (Table 14).

The values obtained for GSSG in this experiment were up to almost 20 times higher than the highest levels quoted by Eggleston and Krebs (25), and approximated 25 to 30% of the total glutathione level, as compared to the estimated 2% reported by Wendell (30). This discrepancy may have been due to extensive nonenzymatic oxidation of GSH during the preparation of the samples, as the GSH assays were run 6 to 8 hours after livers were excised. Also, some of the total glutathione levels were slightly less than their corresponding GSH values, in which case the GSSG levels were recorded as zero. Although the absolute values of GSSG reported in this experiment are most likely too high, the relative values are thought to be reliable data, since oxidative changes should be similar in each of the paired samples.

The Effect of Dietary Sulfate and In Vitro Sulfate and GSSG on G6PDH Activity

G6PDH assays were run on the supernatant preparations used for the GSSG determinations, and GSSG and $SO_4^=$ were added to identical reaction mixtures in order to determine whether either of the substances could prevent the variable effect of dietary sulfate on G6PDH activity.

Table 14

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Levels of Total, Reduced and Oxidized Glutathione in Rat Liver when the Total Dietary Cysteine Was Kept Constant

% Dietary Sulfate	Total Glutathione	Reduced Glutathione	Oxidized Glutathione	
		mg/g protein[a]		
0.0002	54 ± 39[b]	37 ± 29	17 ± 13	(5)[c]
0.02	53 ± 40	38 ± 28	15 ± 13	(5)
0.42	50 ± 33	34 ± 26	16 ± 11	(5)

[a]Values are means ± SD.

[b]All means followed by asterisk are significantly different ($P < 0.1$) by the t-test for paired comparisons.

[c]Numbers in parentheses indicate the number of animals used.

Physiological Levels of $SO_4^=$ and GSSG

The GSSG level used in the reaction mixture was 0.027 mM, which, as mentioned previously, is about half the physiological level, as estimated by Wendell (30). This level was shown in a preliminary test to reduce G6PDH activity by 35%.

Physiological levels of free sulfate were estimated by a radioassay technique. The level of sulfate in a 5 ml sample of rat liver supernatant was found to be about 0.34 µg (Figure 4), which approximates a physiological concentration of 1.0 µg $SO_4^=$/3 ml. However, 5.0 µg $SO_4^=$/3 ml reaction mixture was used in this experiment. This level resulted in a 27% reduction in G6PDH activity in a preliminary test.

G6PDH Activity

When total sulfur in the diet was kept constant by methionine supplementation, a decrease in G6PDH activity was observed at the $SO_4^=$ levels above and below the optimum level of dietary sulfate (Table 15). Although this difference was large, it was not significant, possibly due to the large variation within the 0.02% $SO_4^=$ group. A significant increase ($P < 0.1$) in G6PDH activity between the low and high sulfate diets was observed. Neither GSSG nor $SO_4^=$ eliminated the differential effect of dietary sulfate on G6PDH activity, although they both reduced the effect.

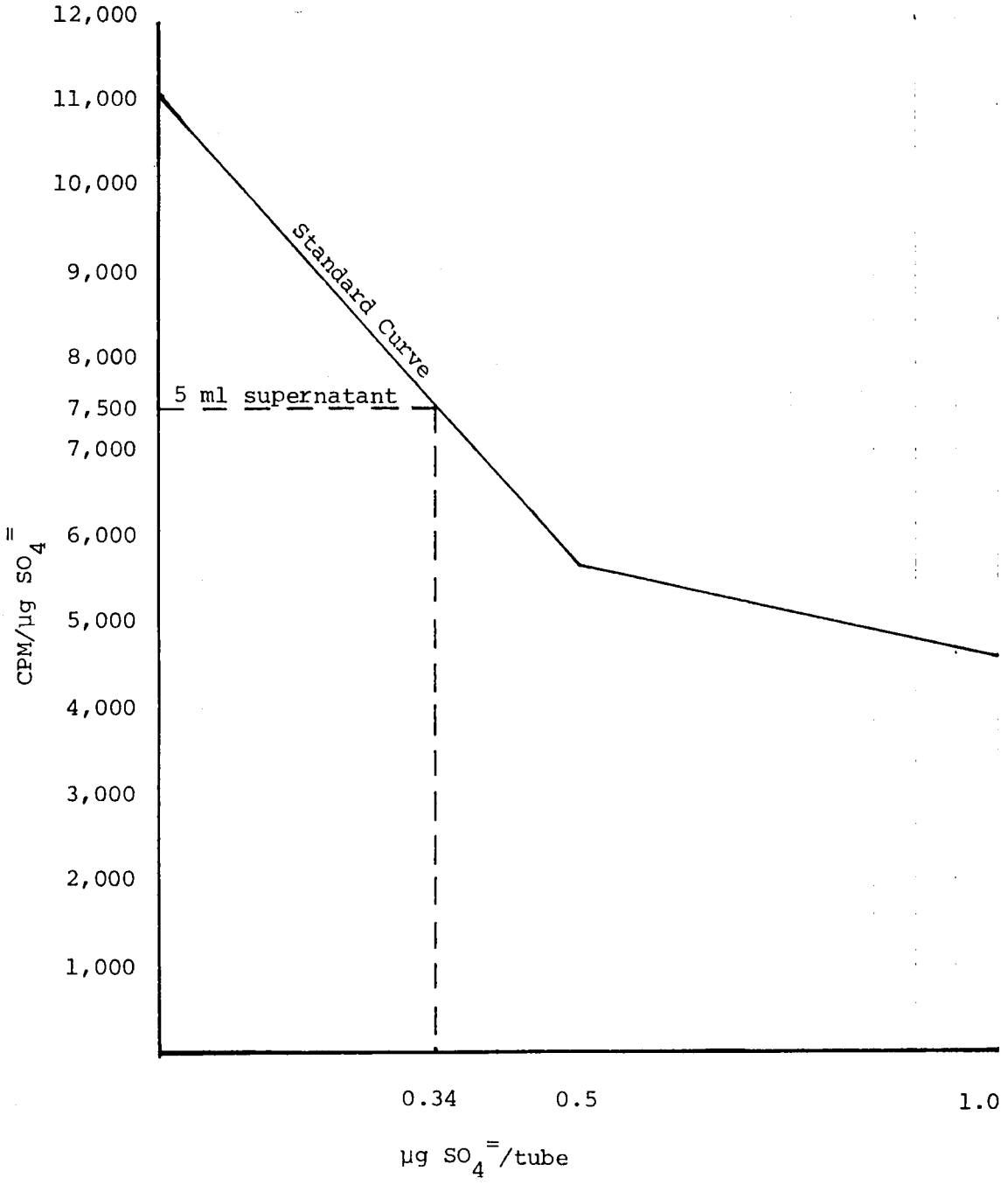

Figure 4. Standard curve for the calculation of $SO_4^=$ level in 5 ml of rat liver supernatant.

Table 15

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Activity of Rat Liver Glucose-6-Phosphate Dehydrogenase and the In Vitro Effect of Oxidized Glutathione and Sulfate when the Total Dietary Sulfur Was Kept Constant by Varying the Level of Methionine

% Dietary Sulfate	% Dietary Methionine	Glucose-6-Phosphate Dehydrogenase Activity	
		µmoles NADPH/min[a] g protein	
0.0002	0.66	10 ± 8*[b]	(5)[c]
with GSSG		9 ± 8	
with $SO_4^=$		10 ± 8	
0.02	0.62	29 ± 32	(5)
with GSSG		18 ± 16	
with $SO_4^=$		20 ± 17	
0.42	0.00	14 ± 6	(5)
with GSSG		12 ± 6	
with $SO_4^=$		12 ± 9	

[a]Values are means ± SD.

[b]All means followed by asterisk are significantly different ($P < 0.1$) by the t-test for paired comparisons.

[c]Numbers in parentheses indicate the number of animals used.

When total dietary sulfur was kept constant by cysteine supplementation, there was again a noted, but not significant, decrease in G6PDH activity at high and low sulfate levels, as compared to the optimum level (Table 16). Again, addition of GSSG or $SO_4^=$ to the reaction media did not eliminate the differential effect of dietary $SO_4^=$ on G6PDH activity.

When dietary cysteine was kept constant, there was no apparent difference between the optimum sulfate and the high sulfate diets, but a slight decrease in activity was still observed at the low sulfate level (Table 17). Again, neither GSSG nor $SO_4^=$ eliminated the differential effect of dietary sulfate on G6PDH activity.

Table 16

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Activity of Rat Liver Glucose-6-Phosphate Dehydrogenase and the In Vitro Effect of Oxidized Glutathione and Sulfate when the Total Dietary Sulfur Was Kept Constant by Varying the Level of Cysteine

% Dietary Sulfate	% Dietary Cysteine	Glucose-6-Phosphate Dehydrogenase Activity	
		μmoles NADPH/min[a] g protein	
0.0002	0.530	16 ± 8[b]	(5)[c]
with GSSG		13 ± 7	
with $SO_4^=$		16 ± 8	
0.02	0.505	20 ± 14	(5)
with GSSG		18 ± 14	
with $SO_4^=$		21 ± 16	
0.42	0.000	14 ± 6	(5)
with GSSG		12 ± 6	
with $SO_4^=$		11 ± 9	

[a]Values are means ± SD.

[b]All means followed by asterisk are significantly different ($P < 0.1$) by the t-test for paired comparisons.

[c]Numbers in parentheses indicate the number of animals used.

Table 17

Effect of Feeding Different Levels of Dietary Inorganic Sulfate on the Activity of Rat Liver Glucose-6-Phosphate Dehydrogenase and the In Vitro Effect of Oxidized Glutathione and Sulfate when the Total Dietary Cysteine Was Kept Constant

% Dietary Sulfate	% Dietary Cysteine	Glucose-6-Phosphate Dehydrogenase Activity	
		µmoles NADPH/min[a] g protein	
0.0002	0.505	16 ± 6*[b]	(5)[c]
with GSSG		13 ± 5**	
with $SO_4^=$		18 ± 9	
0.02	0.505	20 ± 14	(5)
with GSSG		18 ± 14	
with $SO_4^=$		21 ± 16	
0.42	0.505	21 ± 7*	(5)
with GSSG		19 ± 7**	
with $SO_4^=$		22 ± 7	

[a] Values are means ± SD.

[b] All means followed by asterisk are significantly different ($P < 0.05$) and all means followed by a double asterisk are significantly different ($P < 0.1$) by the t-test for paired comparisons.

[c] Numbers in parentheses indicate the number of animals used.

CHAPTER V

DISCUSSION

Previous investigations in this laboratory have documented that metabolic alterations, particularly in carbohydrate and lipid metabolism, occur in rats fed diets containing inorganic sulfate at levels above or below the established optimum level of 0.02%. Data presented by Bittle[1] indicate that inorganic sulfate, when fed at levels above or below the 0.02% level, cause a decrease in the activity of acetyl CoA carboxylase in livers of rats fed the CTS-VM and CC diets. Acetyl CoA carboxylase is considered the rate-limiting enzyme of fatty acid synthesis in animal tissue, thus an effect on this enzyme suggests an effect on the rate of fatty acid synthesis. Fatty acid synthesis requires the presence of reducing equivalents, particularly NADPH; therefore, it is reasonable to assume that NADPH is produced at an increased rate when the rate of fatty acid synthesis increases. Four enzymes are known to generate NADPH, including malic enzyme, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Bittle[1] found higher liver malic enzyme activities at the low and high sulfate levels than at the optimum level for rats

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[1]Bittle, op. cit.

consuming the CTS-VM and CTS-VC diets, but highest activity at the optimum level on the CC diets. However, none of these differences were significant. Previous investigations have shown that isocitrate dehydrogenase did not fluctuate with fatty acid synthesis (68). Thus, neither of these enzymes appears to be the provider of the higher levels of NADPH needed at the optimum sulfate level of the CTS-VM fed rats because of the increased rate of fatty acid synthesis. This study was designed to determine if glucose-6-phosphate dehydrogenase activity varied with fatty acid synthesis and, thus, could be the provider of additional NADPH when fatty acid synthesis rates were elevated at the optimum sulfate level. The activity of 6-phosphogluconate dehydrogenase was not tested since it is involved in the pentose phosphate pathway of which glucose-6-phosphate dehydrogenase is the rate-limiting enzyme, so any changes in G6PDH activity would result in changes in 6PGDH activity as well.

Preliminary studies on the effect of dietary sulfate showed a significantly higher G6PDH activity in rats fed the lower sulfate level of the CTS-VM diets and no differences between the rats fed the CC diets (Tables 8 and 9, pp. 64, 65). These results did not correspond to the changes in fatty acid synthesis suggested by Bittle.[2] However, the rats used in this study were larger than those used by Bittle. When rats

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[2]Bittle, op. cit.

of a similar size as those of Bittle were used, and when the experimental diets were fed for longer periods of time, a higher G6PDH activity was observed at the optimum sulfate level than at the high and low sulfate levels for the CTS-VM diets (Table 15, p. 76), as shown in Figure 5. Although the differences were not significant, the correspondence between these data and that of acetyl CoA carboxylase further support the postulate that a difference might be shown if larger sample sizes were used. There were no significant differences between the G6PDH activities on the CTS-VC diets (Table 16, p. 78) as was observed for acetyl CoA carboxylase by Bittle.[3] When dietary cysteine was kept constant, there was a significant increase ($P < 0.05$) in G6PDH activity from the low to the high sulfate levels (Table 17, p. 79), as shown in Figure 5. It may be concluded that, for the CTS-VM diets but not the cysteine supplemented diets, G6PDH—and consequently 6PGDH—appear to be the providers of increased NADPH levels required for the increased fatty acid synthesis observed at the optimum level of dietary sulfate.

An attempt was made in this study to determine the mechanism by which dietary sulfate affected G6PDH activity. Two possible mechanisms were investigated.

1. The reversal by GSSG of the inhibition of G6PDH by high NADPH/NADP ratios might account for the sulfate

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[3]Bittle, op. cit.

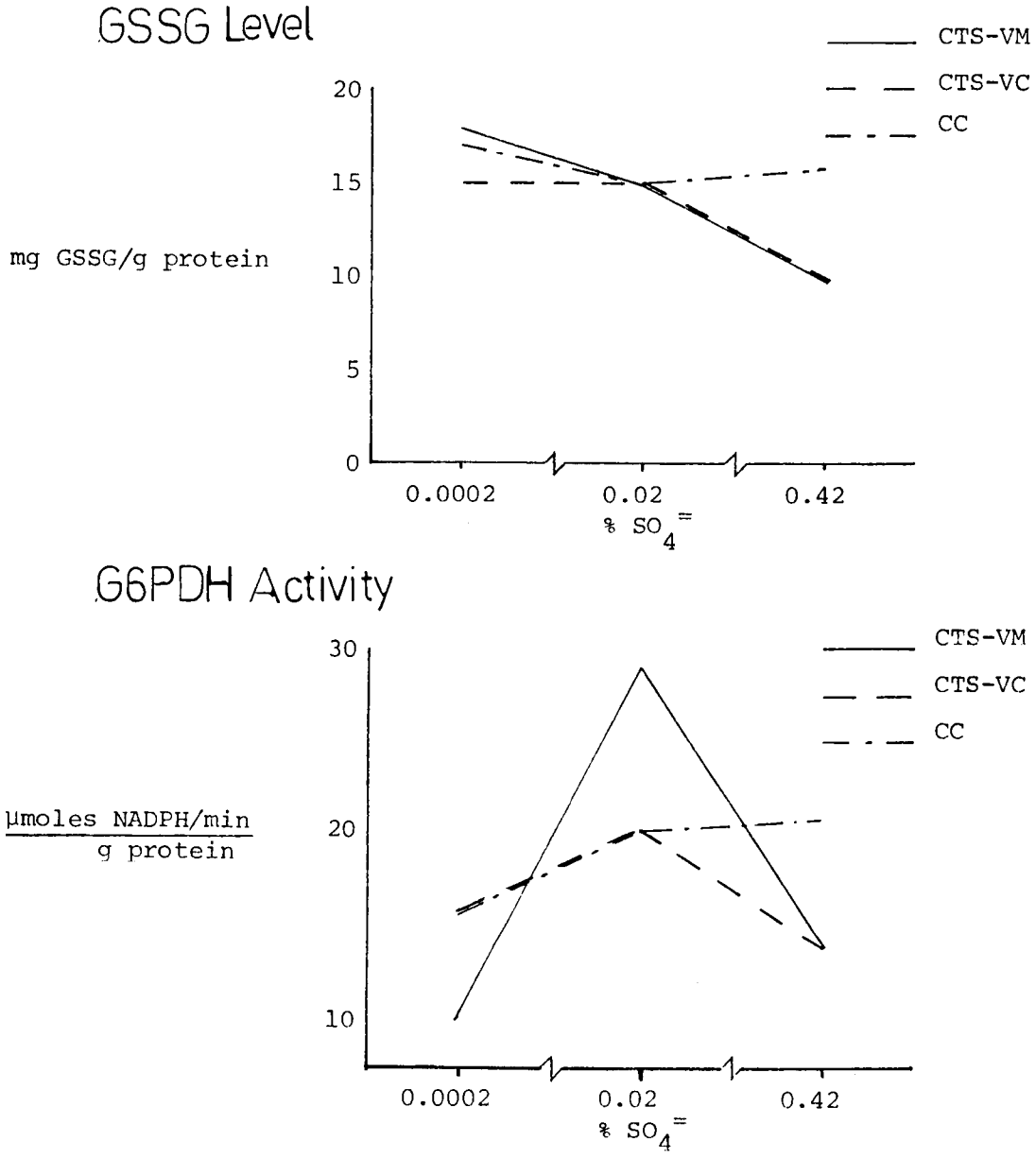

Figure 5. Effect of dietary sulfate on oxidized glutathione levels and glucose-6-phosphate dehydrogenase activity. A comparison of the constant total sulfur-variable methionine, the constant total sulfur-variable cysteine and the constant cysteine diets.

effects if dietary sulfate influences the tissue levels of GSSG.

2. Sulfate itself could be an allosteric effector of G6PDH.

The testing of these two postulates involved the measurement of GSSG-R and GSH-Px activities (experiment 2) of GSSG levels and of G6PDH activity, alone and in the presence of estimated physiological concentrations of GSSG and $SO_4^=$ (experiment 3).

Both GSSG and $SO_4^=$ were shown to reduce the differential effect of dietary sulfate on G6PDH activity when total dietary sulfur was kept constant by methionine supplementation, as shown in Figure 6. This observation suggests that these substances might be involved in the G6PDH effect; however, they did not completely eliminate the effect, thus, other factors must be involved.

These in vitro effects cannot be interpreted as possible in vivo effects unless differences in tissue levels of these substances among rats fed the various diets can be demonstrated and that these differences correspond to the differences in G6PDH activities. A method for $SO_4^=$, sensitive enough to detect differences in tissue $SO_4^=$ levels between rats fed the different diets, has not yet been developed. GSSG levels were found to decrease with increasing dietary sulfate on the CTS-VM diets (see Table 12, p. 70). At the sulfate level above the optimum level, this decrease

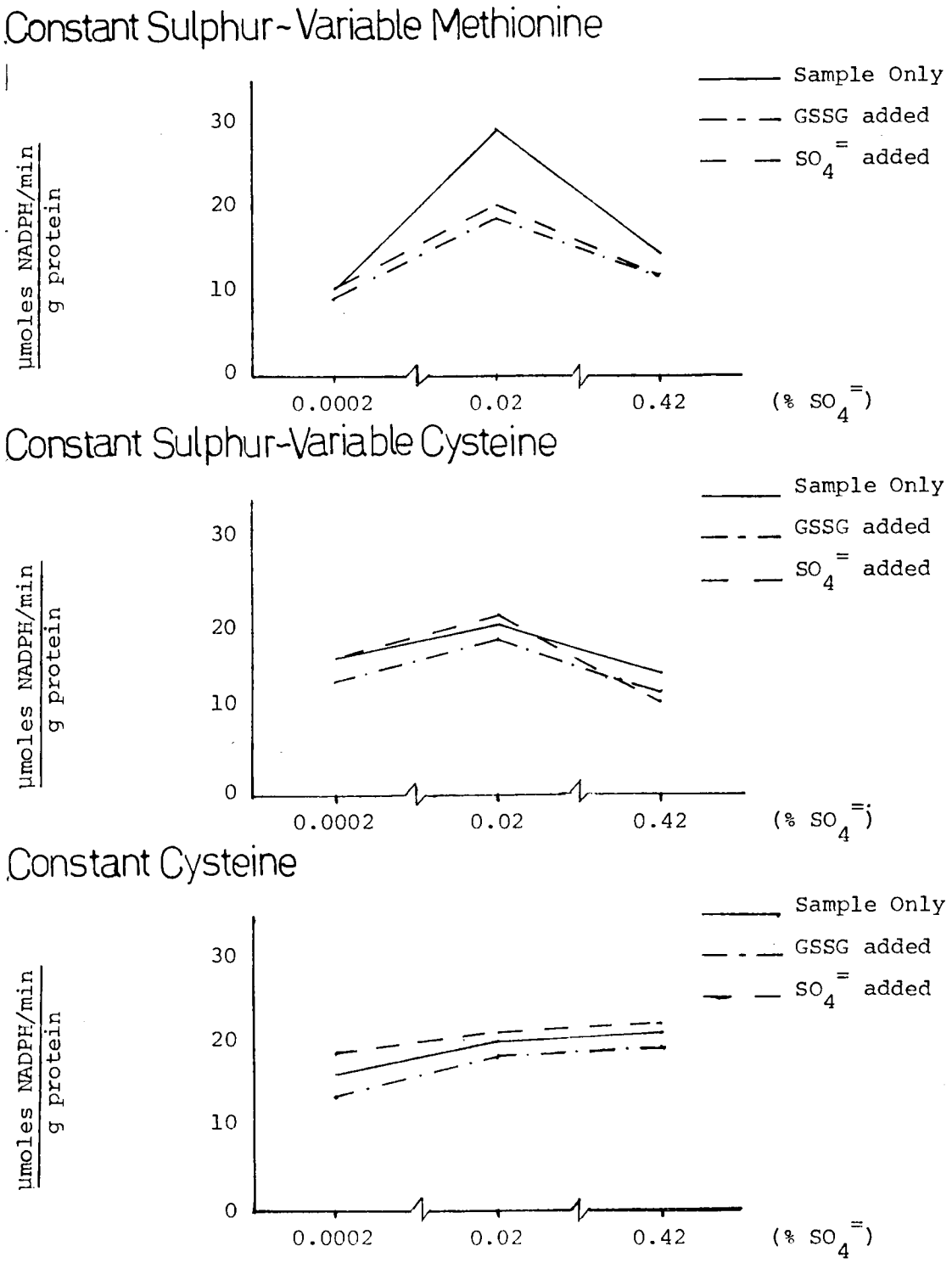

Figure 6. In vitro effect of oxidized glutathione and inorganic sulfate on glucose-6-phosphate dehydrogenase activity.

corresponded to a decrease in G6PDH activity (see Figure 7). Thus, the decrease in GSSG levels at the high sulfate level may cause a decrease in G6PDH by decreasing its de-inhibition of G6PDH demonstrated in vitro by Eggleston and Krebs (25). However, GSSG levels cannot explain the decrease in G6PDH activity at the low sulfate level and the decrease in GSSG levels with increased dietary sulfate may simply be a result of the low levels of methionine and cysteine in the high sulfate diet. Also, the in vitro tests should be repeated in the presence of high NADP/NADPH ratios before concluding that GSSG de-inhibition of G6PDH explains the G6PDH effect. Figure 7 also clarifies the relationship between GSSG-R and GSH-Px activities and GSSG levels. Since both GSSG-R and GSH-Px activities increase with increasing dietary sulfate up to the optimum level, they do not explain the corresponding decrease in GSSG levels. However, as the dietary sulfate level increases above the optimum level, the GSSG-R activity continues to increase but the GSH-Px level drops. Since GSSG-R increases the reduction of GSSG as the rate of re-oxidation by GSH-Px decreases, the levels of GSSG would be expected to decrease, as was observed.

As in the case of the G6PDH activities, cysteine supplementation produced different results with respect to GSSG levels than did the methionine supplemented diets. The CTS-VC diets resulted in differences in GSSG levels between rats fed the optimum level of sulfate and the high sulfate

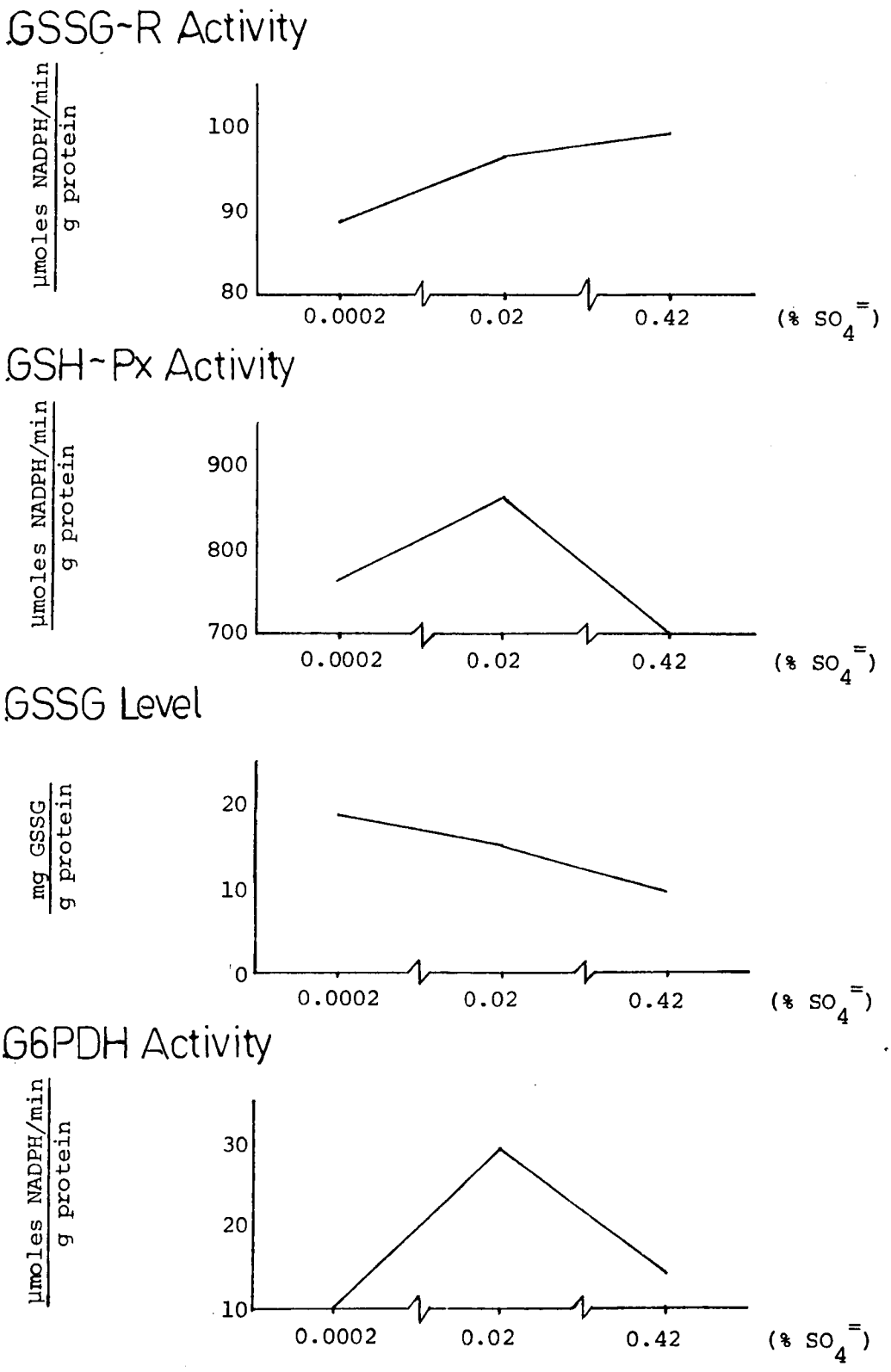

Figure 7. Effect of dietary sulfate on glutathione reductase, glutathione peroxidase and glucose-6-phosphate dehydrogenase activity and on oxidized glutathione levels.

diet only (Table 13, p. 71) and no effect on dietary sulfate was seen in rats fed the CC diets (Table 14, p. 73). As seen in Figure 5 (p. 83), the differences in GSSG levels between rats fed the optimum and high sulfate diets correspond to the observed changes in G6PDH activities. However, as these differences were not significant, no conclusions can be drawn from these data. Also, these observations do not explain the differences between the methionine supplemented and the cysteine supplemented diets. Measurement of tissue $SO_4^=$ levels might contribute to the determination of the reason for this difference.

The decrease in G6PDH activity at a level of dietary sulfate below the optimum level has not been explained by this study. This effect does not appear to be due to a direct effect of $SO_4^=$ on the enzyme since levels of sulfate 5 times greater than the estimated physiological level did not eliminate the differences between the diets, although it may contribute to the effect. Possibly, the theory of Kelly (47), that long-chain fatty acyl CoA derivatives irreversibly inhibit G6PDH, explains the drop in G6PDH activity at low sulfate levels. Assuming that a decrease in lipogenesis would be accompanied by increased levels of fatty acid metabolites, the G6PDH effect could be a secondary effect of the lowered rates of fatty acid synthesis at high and low sulfate levels. Another possible explanation for the

decreased G6PDH activity at low sulfate levels is that a decreased level of sulfate in the tissues could result in a decrease in the synthesis of "active sulfate" or 3'-phospho-adenosine-5'-phosphosulfate (PAPS) which is formed from $SO_4^=$ and ATP. Thus, a decrease in sulfate levels could result, theoretically, in a decreased requirement for ATP. ATP, or purine, synthesis requires 5-phosphoribosyl-1-pyrophosphate (PRPP) of which ribose-5-phosphate, a product of the pentose phosphate pathway, is a component. Therefore, a decreased requirement for ATP could result in a temporary increase in ATP levels, which would have an inhibitory effect on G6PDH activity. The decreased G6PDH activity would result in decreased synthesis of ribose-5-phosphate, which would in turn decrease the levels of PRPP and a decrease in purine synthesis would follow, resulting in a decrease in the levels of ATP.

In conclusion, dietary sulfate appears to have an effect on G6PDH activity and this effect is modified by the levels of cysteine and methionine, or neutral sulfur, in the diet. The mechanism of the effect has not been elucidated. There is evidence that GSSG plays a role at high sulfate levels; however, it has not been proven that sulfate itself is involved in the control of GSSG levels, as the change in GSSG levels could have resulted from changes in the level of neutral sulfur in the diet, since no differences were

observed between the optimum and high sulfate diets when cysteine was kept constant. The decreased G6PDH activity observed, but not proven to be significant, at the low sulfate level in all cases apparently cannot be explained by the levels of free $SO_4^=$ in the tissues—although this needs further investigation. The presence of fatty acid metabolites or a decrease in the utilization of ATP for PAPS synthesis are possible explanations for this effect.

CHAPTER VI

SUMMARY

Alterations in the activity of acetyl CoA carboxylase and, thus, the rate of fatty acid synthesis, have been demonstrated in rats fed diets containing inorganic sulfate at levels above or below the established optimal level of 0.02%. This alteration was not accompanied by a corresponding change in the activity of malic enzyme, a supplier of NADPH, when total sulfur in the diet was kept constant. This investigation was designed to determine if the NADPH needed for the variable rates of lipogenesis was supplied by glucose-6-phosphate dehydrogenase.

When total dietary sulfur was kept constant by either methionine or cysteine supplementation, the activity of G6PDH tended to be higher in rats fed the optimum level of dietary sulfate, as was observed in the case of acetyl CoA carboxylase. When dietary sulfate was greater than the optimum level, there was a decrease in GSSG levels corresponding to the decreased G6PDH levels. This decrease in GSSG levels could be explained by a corresponding increase in glutathione reductase activity as glutathione peroxidase activity dropped. In vitro, GSSG was shown to decrease but not abolish the differential effect of dietary sulfate on G6PDH activity.

When dietary cysteine was kept constant, G6PDH activity increased with the increasing level of dietary sulfate, however there was not a corresponding increase in GSSG levels and in vitro GSSG did not appear to change the G6PDH effect.

In vitro $SO_4^=$ had a similar effect to that of GSSG. It decreased but did not abolish the differential effect of dietary sulfate on G6PDH activity when total dietary sulfate was kept constant with methionine supplementation, but did not appear to change the relationship between dietary sulfate and G6PDH activity in the cysteine supplemented diets.

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