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I am submitting herewith a dissertation written by Ramesh S. Verma entitled "An Investigation of the Effect of Dietary Sulfate on Bile Acid Conjugation and Kinetic Studies of Bile Acyl Transferase in Rat Liver Microsomes." 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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AN INVESTIGATION OF THE EFFECT OF DIETARY SULFATE ON BILE ACID CONJUGATION AND KINETIC STUDIES OF BILE ACYL TRANSFERASE IN RAT LIVER MICROSOMES

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ABSTRACT

This investigation was undertaken to study the effect of varying levels of dietary sulfate on the relative conjugation of ^{14}C-cholic acid with taurine and glycine in rat liver microsomal preparations with a view to evaluating the factors controlling glycocholic:taurocholic acid (G:T) ratio at the cellular level. Pure microsomal preparations, obtained after centrifuging at high speed, were incubated with the optimum concentrations of ^{14}C-cholic acid, glycine, taurine, coenzyme A, magnesium sulfate, ATP and sodium fluoride for 90 minutes at 37°. The radioactive cholic acid conjugates formed were separated with thin layer chromatography and visualized by spraying with phosphomolybdic acid. The G:T ratios were computed by comparing the counts in glycocholate and taurocholate bands.

No diet related differences were observed in the G:T ratio except in rats fed 0.0002% $SO_4^=$ + 0.4% cysteine diet. Omission of coenzyme A from the reaction mixture resulted in alterations of the G:T ratio which were related to the level of sulfate in diets. A deficiency of inorganic sulfate or cysteine (0.0002% $SO_4^=$ + 0.4% cysteine diet and 0.42% $SO_4^=$ diet) caused an increase in the G:T ratio as compared to the G:T ratio observed in rats fed 0.1% $SO_4^=$ + 0.4% cysteine diet and 0.42% $SO_4^=$ + 0.4% cysteine diet. The overall increase in the G:T ratio in reaction mixtures not containing coenzyme A resulted from a decrease of approximately 85% in taurocholate synthesis and only 55% in glycocholate synthesis.

The synthesis of taurocholate was more dependent upon the level of coenzyme A.

Further, the effect of these diets on the level of free coenzyme A in both the mitochondrial and nuclei free fraction of liver homogenates was studied. There was a decrease in the level of free coenzyme A in the rats fed 0.0002% $SO_4^=$ + 0.4% cysteine diet and 0.42% $SO_4^=$ diet. These are the same diets which caused an increase in the G:T ratio. This showed that an increase in the G:T ratio observed after feeding these diets was caused by a deficiency of hepatic coenzyme A and any diet which changes taurine concentration and/or coenzyme A concentration would also tend to change the G:T ratio.

Michaelis constants (Km) of enzyme bile acyl transferase for glycine and taurine and glycine in the presence of small amounts of taurine were determined. Km of the enzyme for glycine was 15 times higher than its Km for taurine. The Km for glycine in the presence of taurine was further increased, thereby showing that taurine acts as a competitive inhibitor of glycine conjugation. From this investigation it is concluded that there is only one nonspecific enzyme, bile acyl transferase, for which taurine is a preferred substrate due to its lower Km, but this enzyme would also catalyze acyl transfer of other structurally similar compounds like glycine and β-alanine. These data have demonstrated that any dietary treatment which alters the level of taurine and/or coenzyme A in the tissue, will also change the G:T ratio; and may be important in the dietary management of serum cholesterol levels.

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CHAPTER I

INTRODUCTION

Previous investigations from the laboratory of the Nutrition Department, The University of Tennessee, Knoxville, have shown that when rats were fed diets containing similar levels of total sulfur, but varying levels of inorganic sulfur as sulfate, more ^{35}S-cysteine was excreted as ^{35}S-taurine by animals fed low levels of inorganic sulfate (0.0002%) (1). At the same time, an increase in the urinary taurine excretion was observed in rats fed diets with low levels of inorganic sulfur as sulfate when the diets were supplemented with the same amount of organic sulfur as cysteine. It was assumed that the changes in taurine excretion should reflect changes in taurine synthesis and tissue saturation. Therefore, the relationship of dietary sulfate and the availability of taurine in the tissues to the relative conjugation of bile acids in the rat was investigated (2). This study demonstrated that the diet which caused the rats to excrete the least amount of cysteine sulfur as urinary taurine also produced the lowest relative conjugation with taurine. However, those rats forced to satisfy their sulfate requirement by the oxidation of cysteine sulfur produced the largest amount of urinary taurine, but did not have the highest relative conjugation with taurine. The data from these studies indicate that the level of inorganic sulfate might be influencing bile acid conjugation by its effect on conjugating enzymes or cofactors.

Several reports from the literature indicate that the bile acids are conjugated with glycine and/or taurine before they are secreted into the bile (3, 4). The enzyme system of rat liver catalyzing the conjugation of free bile acids with taurine and glycine is confined to the microsomal fraction. Isolated microsomes perform this conjugation in the presence of adenosine triphosphate (ATP), Mg^{++} and coenzyme A (CoASH) (5). Bergstrom and Gloor (6) have demonstrated that mainly taurine-conjugated bile acids are formed in a human liver homogenate when both taurine and glycine are available for conjugation with free bile acids. Bremer (7), using rat liver microsome preparations, has also shown the same preference for the conjugation of taurine with free bile acids. It is not known whether these differences in the relative conjugation of glycine and taurine are due to differences in the availability of taurine and glycine or to differences in the specificity of liver enzymes.

In view of these considerations, the present study is designed to investigate the effect of different levels of inorganic sulfate on the relative conjugation of glycine and taurine with cholic acid in rat liver microsome preparations and also the effect of these diets on the level of free coenzyme A in liver. Further, the kinetics of conjugating enzymes will be studied in order to characterize these enzymes.

CHAPTER II

REVIEW OF THE LITERATURE

The major catabolic pathway of cholesterol in mammals is the formation of bile acids (8). This conversion takes place in the liver and the primary bile acids formed are cholic acid and chenodeoxy cholic acid. The bile acids are conjugated with glycine and taurine in liver and then secreted into the bile (3, 4). In the intestine, microorganisms transform some of these primary bile acids into secondary bile acids such as deoxycholic acid and lithocholic acid. The major portion of all bile acids is absorbed through the intestine and returned to the liver via portal blood (9).

Biosynthesis of Bile Acids

The first step in the formation of bile acids is the 7α hydroxylation of cholesterol. The enzyme cholesterol 7α-hydroxylase is located in the microsomal fraction of rat liver and requires a thermostable thiol cofactor, oxygen and NADPH. This reaction is inhibited by carbon monoxide and reactivated by monochromatic light at 450 nm and, therefore, should be cytochrome P-450 dependent (10, 11). The dehydrogenation of 7α-hydroxycholesterol requires NADP and is catalyzed by the microsomal fraction. 7α-hydroxycholest-4-en-3-one is the common intermediate in the formation of cholic acid and chenodeoxy cholic acid (12). The pathway to cholic acid involves the hydroxylation of 7α-hydroxycholest-4-en-3-one at the 12α-position to form 7α,12α-dihydroxy-cholest-4-en-3-one. This reaction is

also catalyzed by the microsomal fraction and requires NADPH (13). The formation of 5β-cholestan 3α,7α,12α triol from 7α,12α-dihydroxycholest-4-en-3-one involves the saturation of the nuclear double bond and the reduction of the 3-ketone. These reactions are catalyzed by two soluble enzymes and require NADPH (14). The mechanism of degradation of the side chain to complete the formation of bile acid involves ω-oxidation at position 26. This reaction is catalyzed by the mitochondrial fraction of rat liver homogenate. Staple *et al.* (15) demonstrated that NAD was necessary for the formation of 3α,7α, 12α-trihydroxy-5β-cholestan-26-oic acid with a dehydrogenase from the supernatant fraction. The next step involves β-oxidation of the side chain at position 24 to form 3α,7α,12α,24 tetrahydroxy-5β-cholestan-26-oic acid. Hydroxylation at position 24 is catalyzed by enzymes in the mitochondrial fraction supplemented with the supernatant fluid (16). Masui and Staple (16) showed that 3α,7α,12α,24 tetrahydroxy-5β-cholestan-26-oic acid could be converted to cholic acid with the 105,000 x g supernatant fraction supplemented with NAD or NADP.

A different metabolic pathway for the biosynthesis of chenodeoxy cholic acid has been proposed by Ayaki and Yamasaki (17). According to these authors, the rat liver mitochondria can cleave the side chain of 7α-hydroxycholesterol giving 3β,7α-dihydroxychol-5-enoic acid Then this unsaturated bile acid is converted to chenodeoxycholic acid which can be further oxidized to a trihydroxy cholanic acid, probably α-muricholic acid.

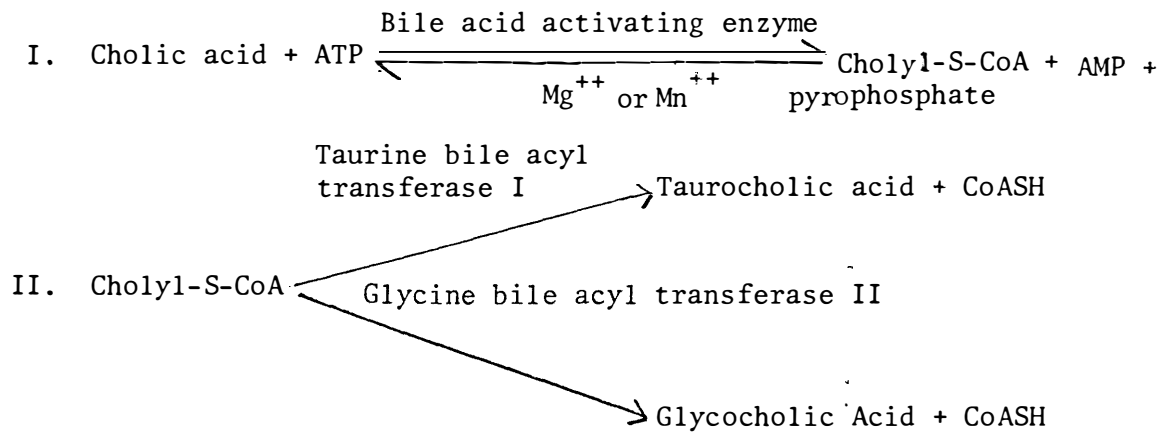
The hepatic bile acid synthesis is regulated by the amount of bile acid returning to the liver via the entero-hepatic circulation

(18). Shefer *et al.* (19) showed that bile acids exerted their regulatory effect via the enzyme cholesterol 7α-hydroxylase provided that hepatic cholesterol synthesis was adequate.

Bile Acids Conjugation

Cholic acid and its derivatives are conjugated with glycine and taurine in the liver before being secreted into the bile as bile acids. The mechanism of the conjugation reaction has been studied by Bremer (5) and Siperstein and Murray (20). They discovered independently of one another that the liver microsomal fraction conjugated cholic acid to glycine and taurine in the presence of adenosine triphosphate (ATP), magnesium (Mg^{++}) and coenzyme A. Bremer (5) obtained evidence which indicated that the reaction proceeded in two steps: first, the activation of cholic acid to cholyl-Co A, then the subsequent transfer of the acyl group to glycine and taurine. On the basis of these results, Bremer (5) proposed that the conjugation was probably catalyzed by three enzymes, one which activated bile acids and two enzymes which transferred the acyl group, one to taurine and the other to glycine. He further demonstrated a much stronger inhibition of taurocholate formation by adenosine monophosphate (AMP) and pyrophosphate than by adenosine diphosphate (ADP) and orthophosphate; therefore, he concluded that the reaction mechanism in this process is identical with that found in acetate activation. In his next study, Bremer (7) carried out experiments indicating that the transfer of the cholyl group from cholyl-coenzyme A to taurine and glycine was irreversible, as the conjugated bile acids were not converted to cholhydroxamic acid in the presence

of microsomes and coenzyme A. On the basis of these studies, Bremer proposed the following reaction schemes:



Schersten (21) studied the conjugation of cholic acid to taurine and glycine in subcellular fraction of human liver, which indicated that the activation of cholic acid took place in the microsomes and that the transfer of bile acyl group to taurine and glycine took place in the lysosomes or peroxisomes. The evidence for placing the bile acyl transferase activity in any of these particles, lysosomes or peroxisomes, is only indirect. For absolute proof, it would be necessary to demonstrate that the substrate involved in the transfer, cholyl-CoA when incubated with a completely pure lysosomal fraction together with glycine and taurine, resulted in the end product conjugates.

Bergstrom and Gloor (6) demonstrated that mainly taurine-conjugated bile acids were formed in human liver homogenates when both glycine and taurine were available for conjugation with free bile acids. The same preference for taurine has been shown by Bremer (7) in rat liver microsomes. He showed that the conjugation of cholic acid with taurine was more than twice as efficient as the conjugation with glycine. The conjugation with glycine was almost totally depressed when enough

taurine was available, although the addition of glycine did not significantly decrease the conjugation with taurine. A different picture was found with rabbit liver microsomes. The conjugation with glycine was of the same order, but only traces of taurine were conjugated. With microsomes from chicken liver, there was a small but significant formation of taurocholic acid. Haslewood and Sjovall (3) have also reported that no glycine-conjugated bile acids could be detected in bird's bile.

The marked influence of taurine administration on the ratio between glycine and taurine conjugated bile acids in man was shown by Sjovall (22). He observed that in man 65 to 75 percent of the total bile acids were glycine conjugates. Taurine feeding (1.0 g x 3 daily) markedly increased the proportion of conjugated bile acid and after 5 days, 95 percent of the bile acids were taurine conjugates; however, feeding glycine (7.0 g x 3 daily) for 12 days did not change the bile acid conjugation pattern of the normal subjects. Feland *et al.* (2) demonstrated that either high or low levels of dietary inorganic sulfate resulted in alterations of the glycocholic to taurocholic acid ratio (G:T ratio) in the extracts of the jejunum-ileum section of the small intestine. The lowest G:T ratio was obtained in rats fed a diet containing 0.1% of inorganic sulfate and 0.4% of added cysteine. The highest G:T ratio was measured in those rats fed a diet containing 0.42% of inorganic sulfate and no added cysteine. Avery and Lupien (23) showed that the synthesis of cholic acid conjugates in liver homogenates of vitamin B-6 deficient rats was significantly greater than in the liver homogenates of the control animals. They postulated

that pyridoxal-PO_4 competitively binds with ε-amino group of lysine at the active sites of the enzymes required for conjugation of cholic acid with glycine and taurine.

Schersten (24) believes that the ability of the liver homogenate to conjugate cholic acid with taurine and glycine is a sensitive test of liver function and that changes in pattern of conjugation occur in diseases affecting the liver. He demonstrated a significant reduction in synthesis of total bile acid conjugates in liver homogenates of patients with obstructive jaundice. He further showed, in cases of obstructive jaundice of short duration, that the synthesis of bile acid conjugates was inhibited directly by the influence of the retained conjugates on the reaction (product inhibition) and indirectly by the stimulation of mitochondrial ATPase activity by retained bile components, causing an increase in the hydrolysis of ATP which results in a relative lack of energy necessary for the conjugation reaction.

Garbutt *et al.* (25) observed a marked increase in relative conjugation of primary and secondary bile acids with glycine and taurine in patients with a variety of ileal disorders. They suggested that the deconjugation of bile acids occurred in areas of regional stasis. The increased amount of unconjugated bile acids presented to the hepatic microsomes could stress the relatively limited amounts of taurine available to the conjugating system. Taurine availability in those patients who had a variable reduction in small intestinal surface area could be reduced further by impaired absorption of dietary taurine. They further demonstrated that oral administration of a taurine load enhanced the relative conjugation of bile acids with

taurine, resulting in at least a four fold reduction in the glycocholic to taurocholic acid ratio.

Interrelationship Between Bile Acids and Cholesterol Metabolism

There is an important interrelationship between bile acids and cholesterol metabolism. Bile acid solubilizes cholesterol in the intestine by forming a micellar solution. Such micellar solubilization is essential for uptake of cholesterol across the mucosal membrane of the intestinal epithelium (9).

According to the current concepts the liver and small intestine are the two major sources of circulating serum cholesterol (26, 27). There may be at least two ways in which bile acids exert their regulatory effect on the cholesterol biosynthesis. (a) The bile acids interfere directly with the synthesis and/or degradation of the enzyme 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG CoA reductase) (28). This enzyme catalyzes the rate limiting step of cholesterol synthesis, the reduction of HMG CoA to mevalonate. (b) The bile acids act indirectly by increasing the rate and efficiency of cholesterol reabsorption. The increased amount of cholesterol thus transported back to liver could cause the decrease of HMG CoA reductase activity. This effect could be similar to the one observed with cholesterol fed animals.

Dietschy (26) has reported that in the intestine the rate of cholesterogenesis from acetate varies inversely as a function of luminal bile acid concentration, i.e., the bile acids directly affect the activity of enzyme HMG CoA reductase and thereby control cholesterol

The relative conjugation of cholic acid with glycine and taurine has been associated with the regulation of serum cholesterol (30, 31). In general, it has been noted that animals which appear resistant to experimentally induced atherosclerosis (rat, dog) conjugate a relatively large portion of bile acid with taurine, whereas the animals which are susceptible to experimentally induced atherosclerosis (rabbit) conjugate few bile acids with taurine (32). Mann *et al.* (30) observed that the feeding of L-cysteine, DL methionine, cystamine, taurine and reduced glutathione lowered the serum cholesterol level in sulfur deficient monkeys. Herrmann (32) suggested that the sulfur containing compounds may depress serum cholesterol by increasing the amount of taurine available for conjugation with cholic acid. Itokawa *et al.* (33) also demonstrated that S-methyl cysteine sulfoxide and S-allyl cysteine sulfoxide found in *Lilliaceae* and *Cruciferae* plants markedly depressed the plasma cholesterol level in rats fed a hypercholesterolemic diet.

Recently, Feland *et al.* (2) showed a relationship between dietary sulfate, glycocholic:taurocholic acid (G:T) ratio and serum cholesterol. They observed a significant increase in both free and total serum cholesterol and highest G:T ratio in rats fed a diet containing 0.42% $SO_4^{=}$. The G:T ratio of the rats fed a diet containing 0.1% of inorganic sulfate and 0.4% of added cysteine was the lowest and correspondingly the total serum cholesterol was also the lowest. These data indicated that the levels of sulfate and of sulfate and taurine precursors in the diet are important in the dietary management of hypercholesterolemia.

Distribution of Taurine

Extensive data demonstrate that taurine is present ubiquitously

in animals (34). Taurine, free or conjugated with bile acids, occurs in a wide variety of mammals. Of the mammals under consideration, least is known about taurine concentrations in human tissues. Taurine levels in adult human liver are intermediate between the high levels in carnivorous animals like the dog, cat and rat and the low or negligible levels in herbivorous animals like the rabbit and guinea pig. In the rat, the highest concentrations of taurine are reported in the heart, muscles, spleen and bone marrow. Further studies (34) indicate that the taurine content of liver in the female rat is about twice that in the male. With the exception of heart tissue, taurine levels in rabbit tissues are low compared with those in other mammals. Taurine is either absent or present in only small amounts in rabbit liver (35) and is not available for conjugation with bile acids in this species. Taurine levels in guinea pig tissues, in general, are similar to those found in the rabbit (34). In particular, taurine appears to be lacking in adult guinea pig liver and this lack is consistent with the fact that almost all conjugates of guinea pig bile acids contain glycine (36).

Biosynthesis of Taurine

Jacobsen and Smith (34) have presented a comprehensive review of the metabolic pathways through which taurine may originate in the biological systems. For purposes of discussion, they distinguish five pathways which are outlined as follows:

Pathway I: methionine--cysteine--cysteinesulfinic acid--hypotaurine--taurine

Pathway II: methionine--cysteine--cysteinesulfinic acid--cysteic acid--taurine

Pathway III: cysteamine/cystamine--intermediates--hypotaurine--taurine

Pathway IV: sulfate--sulfite--intermediates--cysteic acid--taurine

Pathway V: cystine--cysteine disulfoxide--cystamine disulfoxide--hypotaurine--taurine

Pathways I and II are considered to be the main biosynthetic route to taurine in mammalian tissues. A comprehensive review was presented by Whittle[1] on the biosynthesis of taurine and the present review deals mainly with the studies of taurine biosynthesis which have appeared in the literature since her presentation.

Whittle[2] postulated that there might be a pathway other than one involving the decarboxylation of cysteinesulfinic acid. She fed 15% casein diets supplemented with either 0.40% cysteine or 0.53% cysteine or 0.40% cysteine + 0.1% sulfate or 0.42% sulfate or 0.42% sulfate + 0.40% cysteine to rats and measured the activity of enzyme L-cysteine-sulfinic acid carboxy-lyase in the livers. She observed that the animals fed 0.42% sulfate diet exhibited a significant increase in the activity of the enzyme L-cysteinesulfinic acid carboxy-lyase compared to rats fed the other diets. The activity pattern did not parallel the concentration of taurine excreted in the urine (1), or conjugated with bile acids (2) by the rats fed the same diets.

Martin *et al.* (37) demonstrated that the rat was capable of synthesizing the sulfur moiety of taurine from the sulfate. The enzymatic pathway appeared to be similar to that in chicken liver which

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[1]Whittle, B. A. (1970) The influence of dietary sulfate on the excretion of ^{35}S-cysteine sulfur as ^{35}S-taurine sulfur by the rat. Ph.D. Dissertation, The University of Tennessee, Knoxville.

[2]Whittle, B. A. (1974) Liver L-cysteinesulfinite carboxy-lyase activity of rats fed different levels of sulfate and cysteine. Fed. Proceed. 33:682. (Abstr.)

utilizes L-serine and 3'-phosphoadenosine-5'-phosphosulfate (PAPS). The significance of this alternate pathway of synthesis of the sulfur moiety of taurine in mammals may be its regulation by the tissue concentration of sulfur containing amino acids. The availability of the substrates for this reaction, PAPS and serine, is related to cysteine and methionine. Cysteine represses this pathway by its effect on enzyme ATP-sulfurylase, the first enzyme required for the synthesis of PAPS from sulfate. Therefore, in the presence of optimal dietary cysteine, this pathway for the synthesis of the sulfur moiety of taurine from inorganic sulfate becomes inhibited and taurine is synthesized by the degradation of the sulfur containing amino acids.

Sass and Martin (38) reported that the sulfur moiety of taurine may be synthesized *de novo* from inorganic sulfate in chicken liver. The utilization of inorganic sulfate required its conversion to the active form 3'-phosphoadenosine-5'-phosphosulfate (PAPS) after which it was enzymatically transferred to a suitable acceptor molecule by enzyme PAPS-transferase. From this study, Sass and Martin (38) theorized that the sulfur moiety of taurine could be synthesized from sulfate in the chicken by the following pathway:

$$\text{ATP} + SO_4^{=} \xrightarrow[\text{ATP-sulfurylase}]{Mg^{++}} \text{APS} + \text{PPi}$$

$$\text{APS} + \text{ATP} \xrightarrow[\text{APS-kinase}]{} \text{PAPS} + \text{ADP}$$

$$\text{L-Serine} \xrightarrow[\text{Serine-dehydrase}]{\text{Pyridoxal } PO_4} \alpha\text{-Amino acrylic acid} + H_2O$$

$$\alpha\text{-Amino acrylic acid} + \text{PAPS} \xrightarrow[\text{PAPS-transferase}]{\text{Pyridoxal } PO_4} \text{Cysteic acid} + \text{PAP}$$

$$\text{Cysteic acid} \xrightarrow[\text{cysteic acid-decarboxylase}]{\text{Pyridoxal } PO_4} \text{Taurine} + CO_2$$

They further demonstrated that the serine had the highest activity of all the compounds studied, i.e., α-aminobutyric acid, phosphatidyl serine, phosphatidyl ethanolamine, etc., as the acceptor of sulfate from 3'-phosphoadenosine-5'-phosphosulfate (PAPS). Pyridoxalphosphate-dependent serine dehydrase activity was required in the reaction utilizing serine to form taurine. The formation of taurine was preceded by the intermediate being converted to enzyme bound cysteic acid.

Miraglia and Martin (39) isolated and partially purified the enzyme PAPS-transferase and showed that this enzyme was specific for the sulfate moiety of PAPS and did not use inorganic sulfate as efficiently. Hill and Martin (40) measured the activity of the enzyme system which produces taurine from PAPS and serine in chicken liver, for regulation by intermediates of the trans-sulfuration pathway. They observed that the activity of this enzyme system was decreased by L-methionine, S-adenosyl methionine (SAM), homocysteine, cysteine and cysteinesulfinic acid. Cysteic acid, a postulated enzyme bound intermediate of this enzyme system, produced an increase in the activity at low enzyme-protein concentration but had no effect at higher enzyme-protein concentrations.

Tomichek *et al.* (41) studied the effect of a deficiency of certain vitamins on the activity of the enzymes required for the synthesis of the sulfur moiety of taurine from inorganic sulfate. They observed that the deficiency of vitamin A and folic acid caused a decrease in sulfate activation, which might be due to decreased protein synthesis or increased inhibition of the enzymes ATP-sulfurylase and

APS-kinase. A deficiency of vitamin B-6 resulted in a decreased PAPS-transferase activity. Vitamin D deficient chickens showed a lowered rate of taurine synthesis in the liver when $^{35}SO_4^{=}$ was administered orally, whereas no difference was observed in taurine synthesis or PAPS formation *in vitro*.

Coenzyme A Biosynthesis

A combination of enzymatic degradation and enzymatic synthesis carried out mainly by Novelli *et al.* (42) revealed that the structure of coenzyme A contains 1 mole of adenine, 3 moles of phosphate, of which 1 mole is a phosphomono-ester and 1 mole of N-pantothenyl-thio-ethylamine. Mass and Novelli (43) demonstrated that pantothenic acid was synthesized in *E. coli* by the condensation of pantoic acid and β-alanine and the enzyme responsible, pantothenate synthetase (L-pantoate: β-alanine ligase (AMP)) required ATP, K^+ or NH_4^+ and Mg^{++} and during the reaction AMP and inorganic pyrophosphate were formed. The observations by Mass (44) that (a) ^{32}P-inorganic pyrophosphate was incorporated into ATP only in the presence of pantoate, (b) ^{14}C-AMP was not incorporated into ATP under any condition, and (c) pantoyl-hydroxamic acid was formed by incubation of ATP, pantoic acid and enzyme with hydroxylamine, led to the formulation of the following reaction:

$$\text{Enzyme} + \text{ATP} + \text{Pantoate} \underset{}{\overset{K^+,\ Mg^{++}}{\rightleftharpoons}} \text{Enz.-pantoyladenylate} + \text{PPi}$$

$$\text{Enz.-pantoyladenylate} + \beta\text{-alanine} \longrightarrow \text{Pantothenate} + \text{AMP} + \text{enzyme}$$

The main pathway for the conversion of pantothenic acid to coenzyme A was elucidated by Brown (45, 46). The first step, the formation of 4'-phosphopantothenate, is catalyzed by enzyme pantothenate

kinase (ATP: pantothenate 4'-phosphotransferase). The next reaction involves the coupling of 4'-phosphopantothenate and cysteine to yield 4'-phosphopantothenyl cysteine. The coupling enzyme, phosphopantothenyl cysteine synthetase has been purified from mammalian and bacterial sources by Brown (45, 46). The enzymes of rat liver and *Proteus morganii* differ in that the mammalian enzyme will use any nucleoside triphosphate as an energy source, whereas the bacterial enzyme uses CTP specifically. Other substrates that can be used in place of cysteine, but not as effectively, are cystine, β-mercaptoethylamine and α-methyl cysteine. The reaction with β-mercaptoethylamine yields phosphopantetheine as the product. Thus, if the tissue levels of β-mercaptoethylamine were adequate, phosphopantetheine could be formed without the prior formation of phosphopantothenyl cysteine. It appears likely, however, that the latter compound is a normal intermediate since no enzyme has yet been discovered that decarboxylates cysteine to β-mercaptoethylamine. Hogland and Novelli (47) proposed a different metabolic pathway of coenzyme A biosynthesis in animals in which pantothenic acid reacts with cysteine to form pantothenoyl cysteine as the first step followed by decarboxylation to pantetheine which is converted to coenzyme A via phosphopantetheine. Cavallini *et al.* (48) proposed the following pathway for coenzyme A biosynthesis in pigeon liver extracts: pantothenate ——→ pantothenyl cysteine ——→ phosphopantothenyl cysteine ——→ phosphopantetheine ——→ coenzyme A.

To resolve this controversy, complete separation of the phosphorylating enzyme from the cysteine condensing enzyme was required

since the reactions catalyzed by these two enzymes are both reported to proceed only in the presence of ATP and magnesium ions. Abiko (49) succeeded in complete separation of the activities of pantothenate kinase and phosphopantothenyl cysteine synthetase by fractionation of the rat liver extract with protamine sulfate. He showed that when the cysteine condensing activity was separated from the kinase activity, it catalyzed the condensation of phosphopantothenate with cysteine but not of pantothenate with cysteine. Further it was shown that phosphopantothenyl alcohol prepared by enzymatic phosphorylation of pantothenyl alcohol by pantothenate kinase was found to inhibit competitively the condensing reaction between phosphopantothenate and cysteine, whereas pantothenyl alcohol did not affect this reaction. These findings strongly suggest that the phosphate ester moiety of the substrate plays an essential role in the interaction between the substrate and the active site of synthetase. Abiko *et al.* (50) purified rat liver phosphopantothenyl cysteine synthetase by chromatography on various Sephadex gels and dialysis against various buffers of differing ion concentrations and pH. The preparation obtained was free from contamination with inorganic pyrophosphatase and adenylate kinase. They showed a stoichiometric relationship between the amounts of reaction products phosphopantothenyl cysteine, Pi and ADP, indicating the following overall reaction:

Phosphopantothenate + L-cysteine + ATP $\longrightarrow$ phosphopantothenyl cysteine + ADP + Pi

This reaction mechanism is similar to that reported for the formation of glutamine and glutathione. Rozanov *et al.* (51) showed that when

pantothenate was administered parenterally to rats, coenzyme A contents were increased in organs only when ATP, cysteine, thiamin and nicotinate were administered simultaneously. They further showed that 4-phospho-D-pantothenate and especially S-benzoyl-D-pantetheine and 4-pantothenate were more effective in inducing coenzyme A synthesis, possibly due to their closer structural similarity to the coenzyme A.

Another discrepancy between the pathway proposed by Novelli *et al.* (42) and that of Brown (45, 46) concerns the decarboxylation of the cysteine moiety. Hogland and Novelli (47) demonstrated the decarboxylation of pantothenyl cysteine in the course of coenzyme A biosynthesis by rat liver extract, while Brown (45, 46) showed the decarboxylation of phosphopantothenyl cysteine to phosphopantetheine in the crude extract of *Proteus morganii* and rat liver. Abiko (52) purified the enzyme phosphopantothenyl cysteine decarboxylase [4'-phospho-N (L-pantothenyl) L-cysteine carboxy-lyase] from rat liver and showed that the liver contained no enzyme responsible for the decarboxylation of pantothenyl cysteine although phosphopantothenyl cysteine was easily decarboxylated by this purified enzyme to form phosphopantetheine. He further noted that pantothenyl cysteine did not affect the rate of the decarboxylation of phosphopantothenyl cysteine catalyzed by purified decarboxylase, indicating that no interaction occurred between the enzyme and pantothenyl cysteine. Kuwagata (53) confirmed the finding, using ^{14}C-pantothenate, that coenzyme A was biosynthesized in rat liver via 4'-phosphopantetheine.

In the next step of coenzyme A biosynthesis, as proposed by Brown (45, 46), 4'-phosphopantetheine condenses with ATP to form

dephospho coenzyme A, with the liberation of inorganic pyrophosphate. This is the only reversible reaction and is catalyzed by dephospho coenzyme A pyrophosphorylase (ATP: dephospho-CoA 3'-phosphotransferase) and requires ATP specifically as phosphate donor and cysteine for maximum activity. Abiko *et al.* (54) purified dephospho coenzyme A pyrophosphorylase and dephospho coenzyme A kinase from rat liver and confirmed that the final step in coenzyme A biosynthesis was the same as proposed by Brown (45, 46) and Hogland and Novelli (47).

Karasawa *et al.* (55) demonstrated that the level of coenzyme A in tissue was regulated by feed-back inhibition by inhibiting pantothenate kinase. They showed that the pantothenate kinase from rat kidney homogenate was inhibited *in vitro* by coenzyme A and several related compounds, but not by any of the nucleotides containing an adenylate moiety in the structure. *In vivo* studies, in their experiment showed that the coenzyme A levels in the tissues were constant regardless of the amount of pantothenate withheld from the diet, indicating that pantothenate kinase had a binding site besides pantothenate and ATP sites.

The discovery that acyl carrier protein (ACP) contains β-alanine and 2-mercaptoethylamine suggested that this protein might contain bound coenzyme A, since β-alanine and 2-mercaptoethylamine are also components of this coenzyme. Vagelos *et al.* (56) showed that the hydrolysis of ACP by Pronase, a nonspecific proteolytic enzyme, released 1 mole of pantoic acid per mole of protein. In addition, ACP contained 1 mole of organic phosphate per mole of protein. Although both ACP and coenzyme A contained β-alanine, 2-mercaptoethylamine

and pantoic acid, ACP contained a single phosphate and no ribose or adenine. Thus it is clear from this study that ACP does not contain bound coenzyme A, but that it does contain components also found in coenzyme A. The flux of radioactive pantothenate between 4'-phosphopantetheine, coenzyme A and ACP was measured in a pantothenate auxotroph of *E. coli* by Powell *et al.* (57). They showed that coenzyme A was the immediate precursor of ACP and that there was a rapid turnover of 4'-phosphopantetheine prosthetic groups of ACP and of coenzyme A in the exponentially growing cells. That cellular ACP is synthesized at the expense of cellular coenzyme A was demonstrated by Das and Toennies (58) in *Streptococcus faecalis*. They showed that when the medium contained an excess of pantothenate, the cellular contents of coenzyme A and ACP appeared to remain constant during exponential growth, and in a molar ratio of about 4 CoA to 1 ACP. Cellular ACP, once formed, appeared to be stable under these conditions, but coenzyme A was degraded and replaced at the rate of 20% per division period. When the pantothenate was restricted in the medium, initially formed coenzyme A was converted to ACP. However, when the resulting coenzyme A depleted cells were provided with sufficient pantothenate, normal growth was resumed which was preceded by a lag period, during which rapid conversion of ACP to coenzyme A appeared to take place. From this study Das and Toennies (58) suggested a relationship between the demands of viability and the demands of growth. According to them, viability depends upon available ACP; and under chemical stress this is insured at the expense of coenzyme A, whereas growth depends upon available coenzyme A and under conditions of restricted pantothenate supply,

this is provided at the expense of ACP. In their studies with *E. coli*, Alberts and Vagelos (59) also demonstrated the metabolic ascendency of ACP and concluded that ACP concentrations were maintained at the expense of coenzyme A.

Dietary Regulation of Tissue Coenzyme A Levels

Coenzyme A plays a vital role in the oxidation and synthesis of fatty acids, and in their incorporation into lipids. It is also necessary for the oxidation of pyruvate and α-ketoglutarate and is involved in the metabolism of several amino acids. In view of this, any change in the tissue level of coenzyme A would be expected to have widespread effects on the pattern of cellular metabolism. Martynenko and Danchenko (60) showed that in 3 month old rats fed a calorically insufficient diet, the content of coenzyme A in the liver represented only 80% that of normally fed animals and the level remained low up to 24 months. The concentration of coenzyme A in the kidneys and heart was normal at the age of 3 months despite the malnutrition but decreased gradually and at 24 months represented only 84% of the normal level. Tubbs and Garland (61) observed that the proportions of total coenzyme A of liver which were combined with long chain fatty acids and with acetate were increased in rats which were starved, starved and then fed fat, or in which diabetes was induced by alloxan. Yeh and Leveille (62) also observed that the ingestion of a high fat diet significantly decreased the free coenzyme A content of chicken liver. The influence of a short period fast followed the same trend as was observed with feeding a high fat diet. They suggested that an

increased concentration of free fatty acids in tissues would result in an increased rate of fatty acid activation (to CoA derivatives) and could thereby compete with the citrate cleavage enzyme for free coenzyme A. So the depression in hepatic lipogenesis observed under these circumstances could be attributed to an insufficient amount of free CoA for citrate cleavage enzyme resulting in a reduced rate of cytoplasmic acetyl CoA generation. Kataoka (63) and Nakamura *et al.* (64) performed experiments to show the nutritional interrelationship between hepatic coenzyme A and dietary protein or some essential amino acids in young rats. Rats were given either a 18% casein diet (standard diet) or a 5% casein diet (low protein diet) for 4 weeks. Body weight and total hepatic coenzyme A of the standard diet group were markedly increased throughout the feeding period, but such a tendency was not found in the low protein group. The concentration of hepatic coenzyme A in the standard diet group was higher than that of the low protein group during the first 2 weeks of feeding; thereafter, the concentration of coenzyme A in the low protein group was increased at the fourth week. The addition of essential amino acids to the low protein diet increased the liver coenzyme A concentration after 10 days. In further experiments, they found that the hepatic coenzyme A concentration was reduced as compared with control when the methionine diet was administered for 10 days. The effect of lysine and tryptophan deficiencies on hepatic coenzyme A concentration was slight and threonine deficiency hardly affected the hepatic coenzyme A concentration.

Smyth *et al.* (65) reported a decrease in total brain tissue

coenzyme A activity and concentration of rats following a chronic oral administration of 10-20% ethanol *ad libitum*. Ammon *et al.* (66) also observed a decrease in the coenzyme A activity in brain and liver of white mice following an intravenous injection of ethanol. They further demonstrated a short lasting inactivation of coenzyme A in the liver by acetaldehyde administration. In order to differentiate whether this effect was caused by ethanol itself or was mediated through acetaldehyde, in their next study (67) they carried out experiments with homogenates of brain and liver tissues and investigated the effect of ethanol and acetaldehyde on coenzyme A activity. They observed that acetaldehyde lowered the coenzyme A activity in homogenates of brain and liver, whereas ethanol inactivated coenzyme A only in liver homogenate. In the same way, ethanol had no effect on the activity of a solution of coenzyme A in phosphate buffer. Since only liver homogenate contains alcohol dehydrogenase, which is required for the dehydrogenation of ethanol, these authors concluded that the coenzyme A was not inactivated by ethanol itself but through the action of acetaldehyde, which can be formed only in the liver and not in brain tissue.

Hirabayashi and Nose (68) studied the effect of pantothenic acid deficiency in the organs of albino rats. They observed that the levels of pantothenate and coenzyme A were reduced after feeding a pantothenic acid deficient diet for one week. No change in the concentration of coenzyme A was observed in the kidneys and liver of young rats and in the liver of old animals following the injection of pantothenic acid and vitamin B-6 by Martynenko (69). He further demonstrated an increase

in the coenzyme A concentration in the heart of young rats and kidneys of old rats after injecting pantothenic acid and vitamin B-6. Ando (70) showed that when 500 mg of thiamintetrahydrofurfuryl disulfide per day was given to rats for 2 weeks, there was a marked decrease in the hepatic coenzyme A precursors and total pantothenic acid. The values of total pantothenic acid returned to normal and those of coenzyme A precursors to 85% of normal after administration of 5 mg of pantothenic acid daily. He suggested that the lower coenzyme A precursor values observed following excessive administration of the thiamin derivative might be due to an inhibition of phosphorylation of pantothenic acid. Smith *et al.* (71) observed that the concentration of coenzyme A in livers of severely vitamin B-12 deficient ewes was about 2.6 times as great as that in livers of pair-fed animals treated with vitamin B-12. They could not explain the cause of this increase as this elevation in coenzyme A could not be attributed to methylmalonic acid, because the molar concentration of coenzyme A present was more than three times that of methylmalonic acid in both deficient and treated animals.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

The effects of feeding diets containing varying levels of dietary inorganic sulfate and cysteine supplementation on the relative conjugation of cholic acid with taurine and glycine as catalyzed by rat liver microsome preparations were investigated under the conditions of equal concentrations of both substrates in an attempt to confirm previous findings from this laboratory.

Experiment 1

Four groups of randomly selected adult male albino rats of Wistar strain from the stock colony maintained by the Nutrition Department of the University of Tennessee, Knoxville, were used in this investigation. The groups of five animals were fed diets containing different levels of total sulfur as sulfate and different neutral to inorganic sulfate ratios. These diets were composed of a basal diet (Table 1) supplemented to supply the levels of neutral and inorganic sulfate as indicated in Table 2. These diets were the same as those used by Whittle and Smith (1). The rats were housed in groups of five in wire-mesh cages and were given different experimental diets and distilled water *ad libitum*. The diets were fed for 21 days and then the rats were decapitated and their livers removed and homogenized individually for the isolation of the microsomal fraction. The microsomal fraction obtained from each liver was incubated with reaction mixture containing

TABLE 1

COMPOSITION OF BASAL DIET

Component	g/100 g diet
Casein	15.00
Sucrose	30.00
Cornstarch	30.00
Cod-liver oil	2.00
Vegetable shortening[a]	6.00
Vitamin mixture[b]	2.00
Basal salt mixture[c]	1.34

[a]Crisco, Procter and Gamble, Cincinnati, Ohio.

[b]Nutritional Biochemicals Corp., Cleveland, Ohio 44128. Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg vitamin premix): thiamin hydrocholoride 1.0, riboflavin 1.0, niacin 4.5, p-aminobenzoic acid 5.0, calcium pantothenate 3.0, pyridoxine hydrocholoride 1.0, ascorbic acid 45.0, inositol 5.0, choline chloride 75.0, menadione 2.25, biotin 0.020, folic acid 0.090, vitamin B-12 0.00135, α-tocopherol 5.0, vitamin A = 9×10^5 units, vitamin D 1×10^5 units and sufficient glucose to make 1000 g.

[c]Formulated to supply the following amounts of minerals (g/500g basal salt mixture): magnesium carbonate 33.7, sodium chloride 77.4, potassium chloride 125.7, potassium monobasic phosphate 237.8, ferric phosphate 23.0, potassium iodide 0.09, sodium fluoride 0.1, manganese chloride 0.45, aluminum potassium sulfate 0.19 and cupric acetate 0.81.

TABLE 2

VARIATIONS OF BASAL DIETARY MIXTURE

Diet	Additions to Basal Mixture				Levels of Dietary Sulfur		
	$CaSO_4 \cdot 2H_2O$	$CaCO_3$	Cysteine	Non-nutritive Bulk[a]	Inorganic $SO_4^=$	Organic S Expressed As $SO_4^=$	Total S Expressed As $SO_4^=$
	g/100 g diet				%		
1	0	1.34	0.40	11.92	0.0002	0.57	0.57
2	0.18	1.23	0.40	11.85	0.10	0.57	0.67
3	0.75	0.91	0	12.00	0.42	0.25	0.67
4	0.75	0.91	0.40	11.60	0.42	0.57	0.99

[a]Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio.

24-^{14}C-cholic acid, glycine, taurine, coenzyme A, ATP, magnesium sulfate and sodium fluoride. The radioactive cholic acid conjugates were separated with thin layer chromatography using a solvent system containing isopentyl acetate:propionic acid:propanol:water (20:15:10:5 v/v) and visualized by spraying with phosphomolybdic acid. The appropriate bands were removed to scintillation vials containing 2,5-diphenyl oxazole (PPO) in toluene and the radioactivity evaluated with a liquid scintillation spectrophotometer. Relative enzyme activity was expressed as the ratio of glycocholic to taurocholic acid formed under these conditions.

Experiment 2

The results of Experiment 1 showed that there was a significant increase in the incorporation of 24-^{14}C-cholic acid in glycine and taurine in the incubation mixture fortified with coenzyme A as compared with the incubation mixture which was not fortified with coenzyme A. To determine whether a deficiency of dietary sulfate caused the deficiency of coenzyme A, four groups of adult albino rats of the same strain were fed the same diets (Tables 1 and 2) for 21 days and then the rats were decapitated and their livers removed and homogenized for the isolation of mitochondrial and nuclei free fraction. The concentration of free coenzyme A was determined in this fraction by the method of Novelli (72) which involves the formation of acetyl coenzyme A when tissue coenzyme A and acetyl phosphate are incubated in the presence of enzyme phosphotransacetylase. Arsenolysis of acetyl coenzyme A gives free coenzyme A which acts catalytically to form more coenzyme A. The rate of reaction is directly proportional to the

amount of coenzyme A present. The disappearance of acetylphosphate was measured with a Beckman B spectrophotometer at 540 nm.

Experiment 3

The results of Experiment 1 showed that though the concentrations of both glycine and taurine were the same in the incubation mixture there was greater conjugation of 24-^{14}C-cholic acid with taurine than with glycine. To explain the greater preference of cholic acid for taurine when glycine and taurine were available in equal amounts, an experiment was designed to study the kinetics of the enzyme responsible for this conjugation. The Michaelis Constant (Km) of this enzyme for taurine as well as for glycine was studied.

II. METHODS

Isolation of Liver Microsomes

Liver microsomes were isolated from each liver by the method of Rendina (73). Livers were excised and placed in chilled beakers containing cold medium A which consisted of 0.25 M sucrose, 0.01 M potassium chloride and 0.003 M magnesium chloride. Livers were homogenized individually in 10 ml of medium A per gram of tissue using a motor driven Thomas homogenizer with a teflon pestle. The homogenate was centrifuged in metal tubes at 8200 x g for 1 minute in a Lourdes "Betafuge" centrifuge. The supernatant fluid was decanted and again centrifuged for 5 minutes at 40,000 x g in Beckman L5-50 ultracentrifuge. The supernatant fluid was decanted and again centrifuged in cellulose-nitrate tubes at 254,000 x g for 45 minutes in Beckman L5-50 ultracentrifuge. The residual fraction so obtained should contain pure

microsomes. The whole procedure is summarized in the following flow sheet: 10% tissue (w/v) homogenate in 0.25 M sucrose, 0.01 M KCl, 0.003 M $MgCl_2$. Centrifuge for 1 minute at 8200 x g

Centrifuge supernatant fluid for 5 minutes at 40,000 x g

Discard nuclei and cell debris

Discard mitochondria

Centrifuge supernatant fluid at 254,000 x g for 45 minutes

Resuspend microsomes in 4 ml 0.067 M phosphate buffer, pH 7.4

Discard supernatant fluid

Formation of Bile Acid Conjugates

Bremer's (5) method was modified in this laboratory to determine the *in vitro* bile acid conjugation. The microsomal fraction obtained from one cellulose nitrate tube was suspended in 4 ml 0.067 M phosphate buffer, pH 7.4. One ml of this suspension was added to an Erlenmeyer flask containing 4 μmole 24-^{14}C-cholic acid, 4 μmole taurine, 4 μmole glycine, 0.30 μmole coenzyme A, 10 μmole ATP, 0.4 mmole NaF and 2 μmole $MgSO_4$ in 0.5 ml 0.067 M phosphate buffer, pH 7.4. The Erlenmeyer flasks were incubated in a shaking water bath at 37° for 90 minutes. The reaction was stopped by immersing the Erlenmeyer flasks in a boiling water bath for 1 minute. After cooling the Erlenmeyer flasks, the bile acid conjugates were extracted by adding 2 ml 1-butanol and shaking for 15 minutes. The samples were then centrifuged at 715 x g for 10

minutes in International centrifuge model V. The upper layer was collected and evaporated to dryness in a convection oven.

Purification of Standard Glycocholic Acid and Taurocholic Acid

Glycocholic acid and taurocholic acid obtained from Nutritional Biochemical Corporation, Cleveland, were purified by column chromatography. Fifty grams of silicic acid were activated at 110° for 18 hours and then a slurry was made by mixing the silicic acid with chloroform. The slurry was poured into a 3 x 45 cm column to form an adsorbant bed 33 cm high.

Two grams of impure glycocholic acid or taurocholic acid were dissolved in a minimum volume of chloroform:methanol (1:4 v/v) and layered very carefully onto the surface of the column. The bile acids were eluted with chloroform:methanol (1:4 v/v) and collected in a Research Specialties Company fraction collector. The fractions were evaluated for the presence of pure glycocholic acid and taurocholic acid by thin layer chromatography (74, 75). Those fractions which contained pure glycocholic acid were pooled, evaporated to dryness and reconstituted with 1-butanol. The taurocholic acid containing samples were treated in a similar manner.

Separation of Conjugated Bile Acids

The bile acids, glycocholic acid and taurocholic acid, were separated using thin layer chromatography by a modification of the method of Anthony and Beher (74). 0.3 mm thick plates were made with acidic Silica AR-4G. The plates were dried for 5 minutes at room temperature to allow the adsorbant to set and were then activated for 2 hours at 110° in a drying oven. Channels were marked on the plates to delineate adsorbant columns and to prevent a concave solvent front.

The evaporated bile acids were reconstituted with 0.2 ml of 1-butanol containing equal volumes of standard purified glycocholic acid and taurocholic acid. A 100 µl sample of reconstituted bile acids was stripped on the plates 1.7 cm from the bottom of an adsorbant column. After the samples were applied to the plates, the plates were dried in a Freas vacuum oven for 15 minutes at 30 inches of mercury at 38° to insure complete dryness. The plates were allowed to develop by an ascending technique in an equilibrated chamber containing Solvent II as described by Hofmann (75), isopentyl acetate:propionic acid:propanol:water (20:15:10:5 v/v) for the separation of conjugated bile acids. Plates were removed before the solvent front reached the top of the plate. The plates were developed twice and were dried in Freas vacuum oven for 15 minutes between developments. After the second development, the plates were thoroughly dried at 110° for half an hour. After cooling, the bile acid bands were visualized by spraying the plates with freshly prepared 10% phosphomolybdic acid in 95% ethanol and heating at 110° for 15 minutes. The bands were scraped from the plates and quantitatively transferred to scintillation vials containing 10 ml scintillation liquid made by adding 12 g of Packard PPO (2,5-diphenyl oxazole) per liter of toluene. The radioactivity of each sample was evaluated with a Picker Nuclear Liquimat 220, liquid scintillation spectrophotometer. The glycocholic: taurocholic acid (G:T) ratio for each sample was computed by comparing the counts per minute in the taurocholate and glycocholate bands.

Determination of Free Coenzyme A in Mitochondrial Free Homogenate

A 10% liver homogenate was made in 0.1 M Tris buffer, pH 8.0. The homogenate was centrifuged at 28,700 x g for 10 minutes in a Lourdes

"Betafuge" centrifuge to separate nuclei and mitochondria. The supernatant fluid was therefore designated as mitochondrial free homogenate. Free coenzyme A was determined in the mitochondrial free homogenate by the method of Novelli (72). To 0.2 ml of mitochondrial free homogenate, 0.1 ml of 1 M Tris buffer, pH 8.0, 0.1 ml (60 μmole/ml) of acetyl phosphate, 0.1 ml of 0.1 M cysteine hydrochloride, 0.1 ml of (8 units) phosphotransacetylase were added and the mixture diluted to a final volume of 1 ml with 0.1 M Tris buffer, pH 8.0. The reaction mixture was incubated at 28° for 5 minutes and then 0.1 ml of 0.5 M potassium arsenate, pH 8.0 was added. After further incubation for exactly 10 minutes, 1 ml of freshly prepared solution A (made by mixing equal volumes of 4 M hydroxylamine hydrochloride and 3.5 N sodium hydroxide) was added and then the tubes were further incubated for 5 minutes. Then 1.0 ml of distilled water and 3.0 ml of solution B (made by mixing equal volumes of 5% $FeCl_3$, 3 N HCl and 12% trichloroacetic acid) were added. A blank tube and a standard tube containing 0.1 μM coenzyme A were run simultaneously with each determination. The tubes were centrifuged and the absorbance of the supernatant fluid was measured at 540 nm with a Beckman B spectrophotometer.

Calculation:

$$\text{mg CoA/g of tissue} = \frac{\text{Absorbance of Standard}}{\text{Absorbance of Unknown}} \times \frac{\text{Conc. of Standard}}{\text{Weight of tissue}}$$

Micro-Kjeldahl Determination of Microsomal Nitrogen

Microsomal nitrogen was determined by the micro-Kjeldahl procedure as standardized by Willets and Ogg (76). The microsomal fraction was isolated as described before and suspended in 4 ml of 0.067 M phosphate

buffer, pH 7.4. Suspended microsomes (0.5 ml) were transferred to a micro-Kjeldahl digestion flask containing 1.30 ± 0.05 g potassium sulfate, 40 ± 5 mg mercuric oxide and 2.0 ml concentrated sulfuric acid. The flask contents were digested for 4 hours or more until they became clear. The flasks were allowed to cool and approximately 5 ml of distilled water were added to dissolve the solids. The flask contents were quantitatively transferred to a micro-distillation apparatus. A 125 ml Erlenmeyer flask containing 5 ml of 4% boric acid and 4 drops of methylred-bromocresol green indicator was placed under the condenser, with the tip of the condenser extending below the surface. Eight ml of sodium hydroxide-sodium thiosulfate reagent (made by adding 50 g NaOH and 5 g of $Na_2S_2O_3$ .5 H_2O/100 ml water) were added to the still and the mixture steam distilled, until 14 ml of distillate were collected. The contents of the receiving flask were diluted to 50 ml with distilled water and titrated with 0.01 N HCl. The mg of nitrogen were calculated by the following formula:

(ml acid)(N acid)(meq wt of nitrogen in mg) = mg of nitrogen.

III. STATISTICAL ANALYSIS

Data were analyzed by the method of paired comparisons as described by Steel and Torrie (77). The Olivetti-Underwood programma 101 was used for all the computations.

CHAPTER IV

RESULTS

Experiment 1

As there was no method available for measuring the *in vitro* conjugation of bile acids, Bremer's (5) method was modified. For an enzyme to show its maximal activity it must be saturated with substrate. So in the first experiment, optimum concentration of cholic acid and coenzyme A was determined after incubating the microsomal fraction with two levels (4 and 8 μmole/tube) of ^{14}C-cholic acid, different levels of coenzyme A (0.1, 0.15, 0.2, 0.3, 0.4 μmole/tube), taurine (4 μmole), glycine (4 μmole), ATP (10 μmole), sodium fluoride (0.4 mmole) and magnesium sulfate (2.0 μmole) for 90 minutes at 37°. The results of this experiment (Figure 1) show that increasing cholic acid concentration above 4 μmole/tube did not accelerate the formation of ^{14}C-glycocholate or ^{14}C-taurocholate. Maximum formation of ^{14}C-taurocholate occurred when the concentration of coenzyme A was 0.3 μmole/tube. Throughout the range investigated (0.1-0.4 μmole/tube), the concentration of coenzyme A did not affect the formation of ^{14}C-glycocholate. In the next experiment, the optimum concentration of coenzyme A was determined when either glycine or taurine (4 μmole) was present in the reaction mixture, all other conditions remaining the same. This experiment showed (Figure 2) that at 0.1 μmole concentration of coenzyme A, there was a maximum formation of ^{14}C-glycocholate or ^{14}C-taurocholate. In the next experiment, the optimum level of

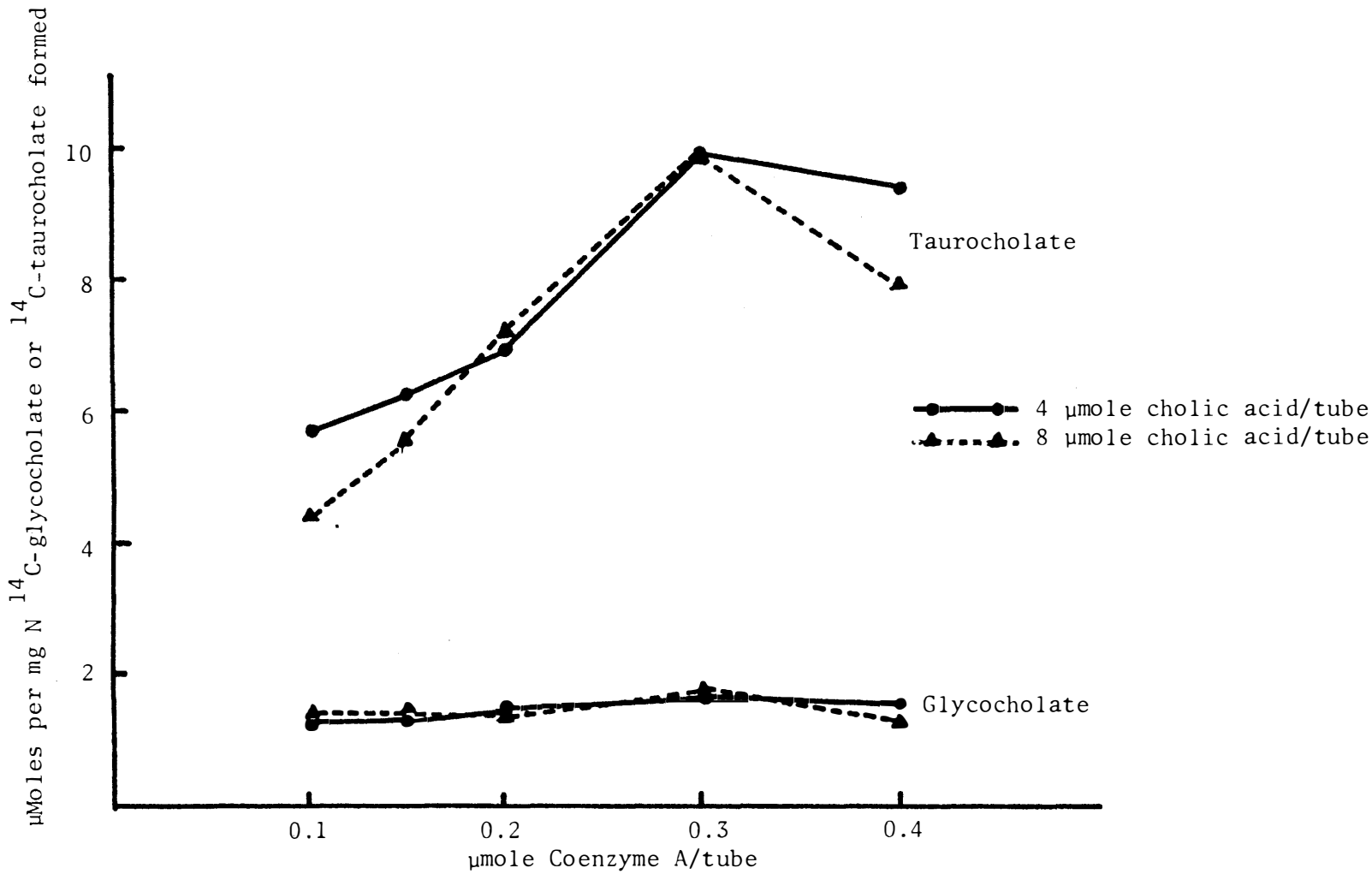

Figure 1. Relationship of ^{14}C-cholic acid conjugation to cholic acid and coenzyme A concentrations when both glycine and taurine are present in the incubation mixture.

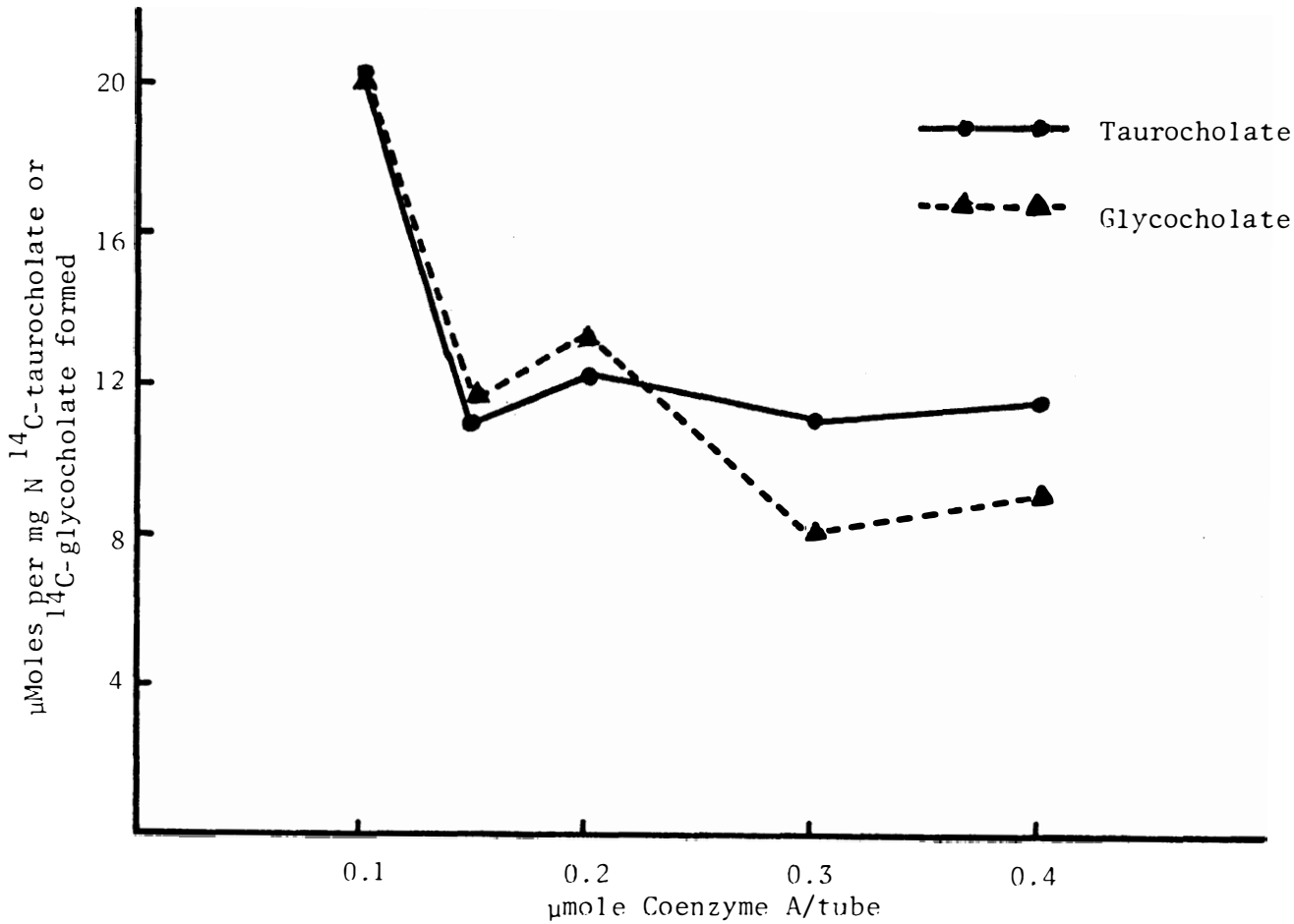

Figure 2. Relationship of ^{14}C-cholic acid conjugation to coenzyme A concentrations when either taurine or glycine is present in the incubation mixture.

glycine or taurine was determined in reaction mixtures containing different levels of either glycine or taurine, all other conditions remaining the same. This experiment showed (Figure 3) that there was a maximum formation of ^{14}C-glycocholate and ^{14}C-taurocholate when the reaction mixture contained 4 μmole of glycine and 4 μmole of taurine respectively. In the next experiment different levels of microsomal preparations were incubated with the optimum concentration of substrate, coenzyme and cofactors (^{14}C-cholic acid 4 μmole, taurine 4 μmole, ATP 10 μmole, sodium fluoride 0.4 mmole and magnesium sulfate 2.0 μmole) for 90 minutes at 37°. A linear relationship (Figure 4) was obtained between microsomal nitrogen and enzyme activity, thereby showing that at these concentrations of substrate, coenzyme and cofactors, the enzymes show maximal activity.

This reaction mixture containing the optimum concentration of all substrates, coenzyme and cofactors was used to measure the glycocholic:taurocholic acid (G:T) ratio in the microsomal preparation of livers of rats fed different levels of inorganic sulfate (Tables 1 and 2, pages 27, 28). The G:T ratio was computed by finding the ratio of ^{14}C-glycocholate to ^{14}C-taurocholate formed based on ^{14}C activity in each fraction. The data from this experiment show (Table 3) that there was no effect of different levels of dietary sulfate on the G:T ratio except in rats fed the lowest level of dietary sulfate (Diet 1). An increase approaching statistical significance ($0.1>P>0.05$) in the G:T ratio was observed in rats fed 0.0002% $SO_4^{=}$ + 0.4% cysteine diet (Diet 1) as compared with rats fed higher levels of dietary sulfate. In order to see whether the changes in G:T ratio were

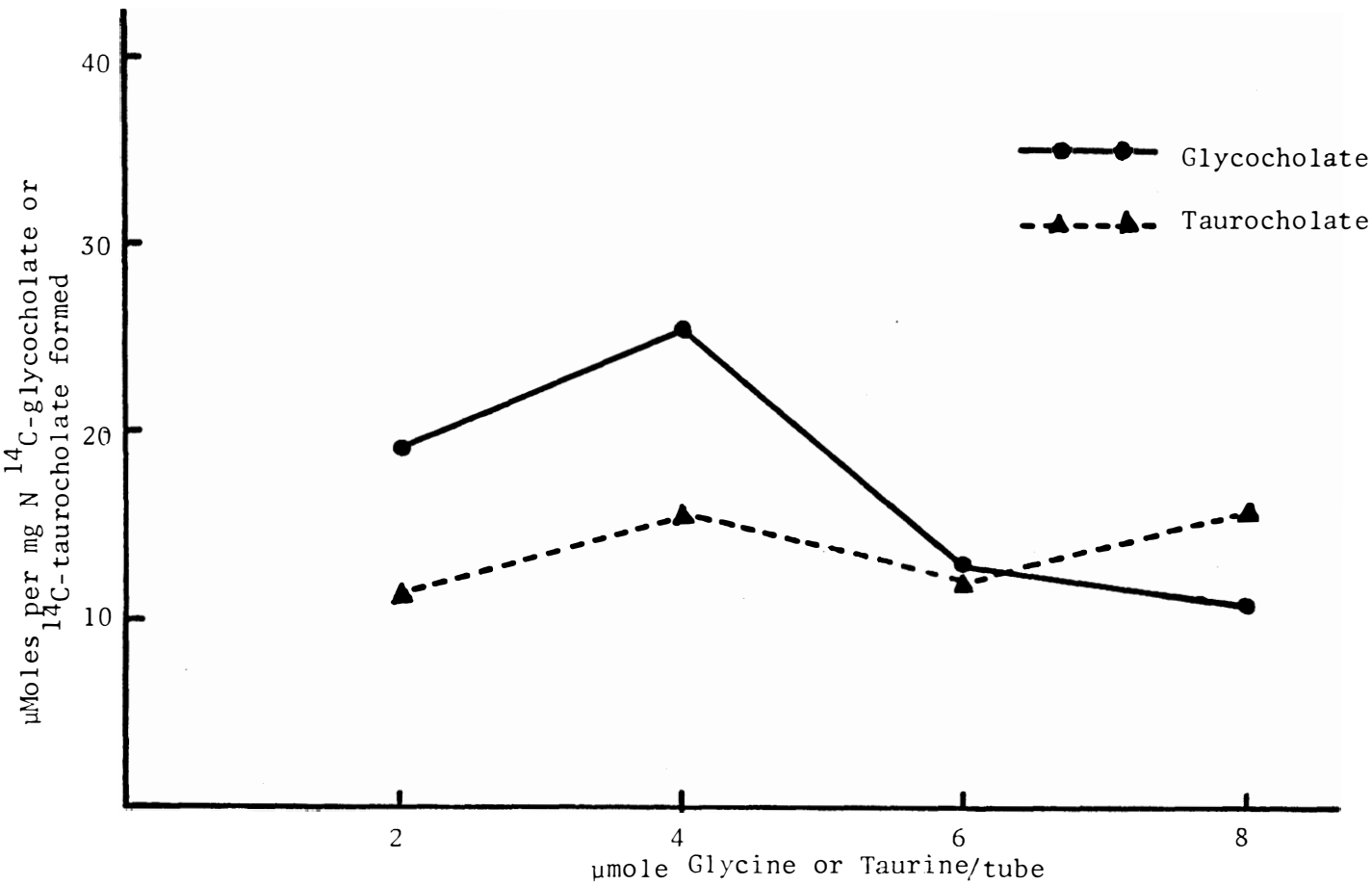

Figure 3. Relationship of ^{14}C-cholic acid conjugation to glycine and taurine concentration.

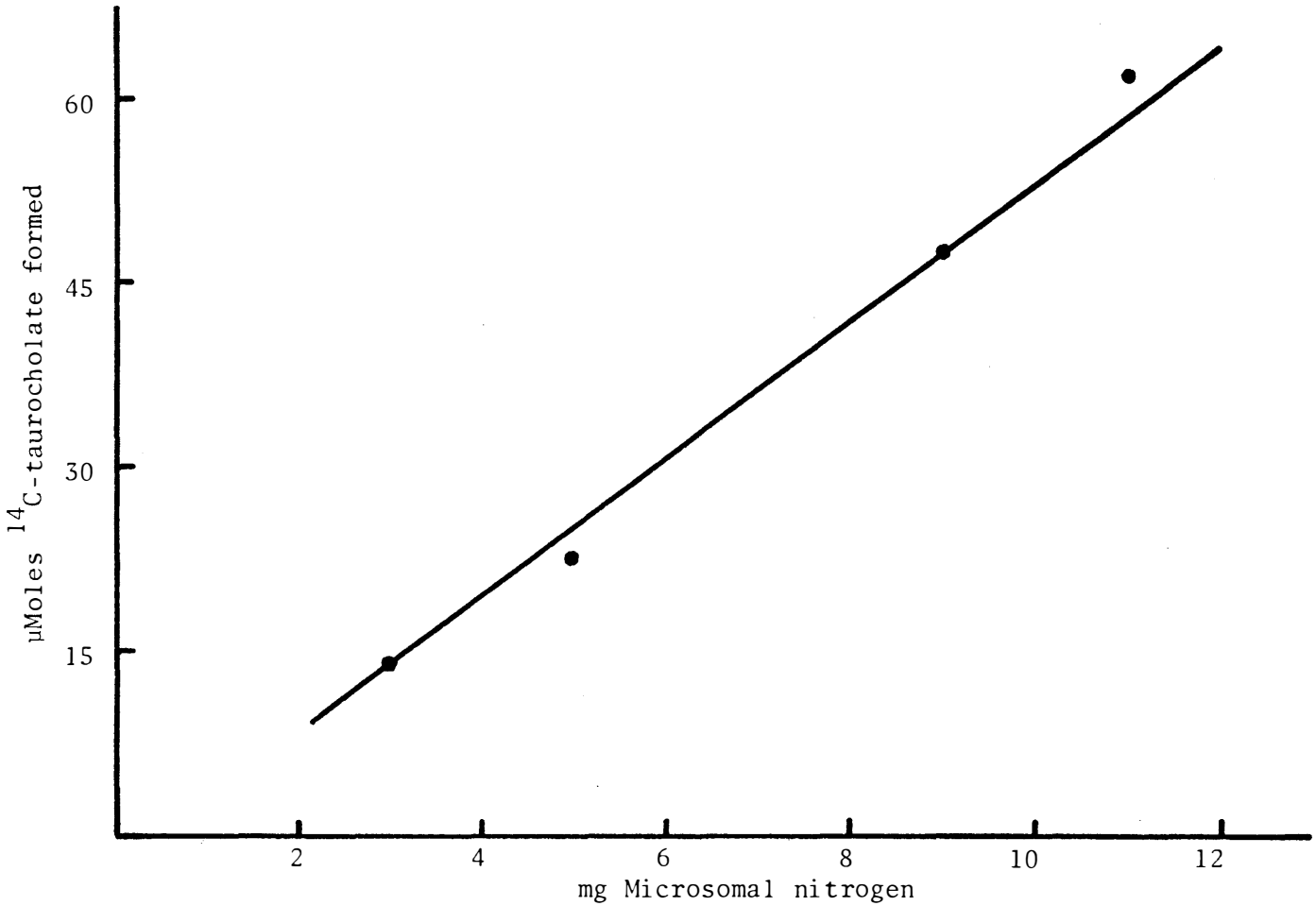

Figure 4. Relationship between microsomal nitrogen and enzyme activity.

TABLE 3

RELATIONSHIP BETWEEN DIETARY SULFATE AND THE G:T RATIO IN RAT LIVER MICROSOMAL PREPARATIONS WITH AND WITHOUT COENZYME A FORTIFICATION

Diet Numbers	Level of Dietary $SO_4^{=}$	Level of Organic S as $SO_4^{=}$ in Diet	Level of Total S as $SO_4^{=}$ in Diet	G:T Ratio $\bar{C}$ CoA	G:T Ratio $\bar{S}$ CoA
	%				
1	0.0002	0.57	0.57	0.21±0.05[a]	0.56±0.17
2	0.10	0.57	0.67	0.11±0.02	0.32±0.09
3	0.42	0.25	0.67	0.11±0.01	0.61±0.27
4	0.42	0.57	0.99	0.12±0.01	0.39±0.15

Statistical Comparisons[b]

$\bar{C}$ CoA		$\bar{S}$ CoA	
1 to 2	$0.1 > P > 0.5$	1 to 2	$P < 0.05$
1 to 3	$0.1 > P > 0.5$	1 to 3	N.S.
1 to 4	$0.1 > P > 0.5$	1 to 4	$P < 0.01$
2 to 3	N.S.	2 to 3	N.S.
2 to 4	N.S.	2 to 4	N.S.
3 to 4	N.S.	3 to 4	N.S.

[a]Values represent average of 10 values obtained from 5 rats ± standard error of the mean.

[b]By paired comparison (77).

related to the level of coenzyme A in the reaction mixture, coenzyme A was omitted from the reaction mixture and the G:T ratio was computed from bile acid conjugation. Changes in the G:T ratio which were related to level of sulfate in the diet (Table 3) were obtained when coenzyme A was omitted from the incubation mixture. A significant decrease in the G:T ratio was observed in liver microsomal preparation of rats fed 0.1% $SO_4^{=}$ + 0.4% cysteine (Diet 2) ($P<0.05$) and 0.42% $SO_4^{=}$ + 0.40% cysteine (Diet 4) ($P<0.01$) diets. This experiment further showed (Table 4) that the overall increase in the G:T ratio observed with omission of coenzyme A from the reaction mixture resulted from a decrease in the taurine conjugation of approximately 85% but only a 55% decrease in glycine conjugation, thereby indicating that the synthesis of taurocholate is more dependent upon the level of coenzyme A than is the synthesis of glycocholate.

Experiment 2

The data presented for experiment 1 demonstrated that the conjugation of taurine with cholic acid was more sensitive to the requirement of coenzyme A than was the conjugation with glycine. It has been well documented (42, 45, 46) that coenzyme A contains mercaptoethylamine, a cysteine derivative, in its molecule. So it was of interest to see whether a deficiency of dietary sulfate caused the deficiency of coenzyme A in the liver. Therefore, in the next experiment, rats were fed the same diets (Tables 1 and 2, pp. 27, 28) for the same period of time (21 days) and the level of free coenzyme A was determined in the mitochondrial and nuclei free fraction of liver homogenate, as this is the only portion of coenzyme A which is available for conjugation reaction.

TABLE 4

EFFECT OF DIFFERENT LEVELS OF DIETARY SULFATE ON THE FORMATION OF ^{14}C-GLYCOCHOLATE AND ^{14}C-TAUROCHOLATE IN RAT LIVER MICROSOMAL PREPARATIONS WITH AND WITHOUT COENZYME A FORTIFICATION

Diet Number[a]	Moles per mg N ^{14}C-Glycocholate Formed		Moles per mg N ^{14}C-Taurocholate Formed	
	$\overline{C}$ CoA	$\overline{S}$ CoA	$\overline{C}$ CoA	$\overline{S}$ CoA
1	5.71±0.76[b]	2.31±0.11[c]	43.15±14.45	6.96±1.14[d]
2	5.78±0.83	2.35±0.27	51.33±12.74	8.32±1.93
3	6.32±0.65	2.15±0.14	62.58±9.60	6.82±1.25
4	5.29±0.99	2.46±0.22	50.36±12.98	14.65±4.21

[a]Diet number corresponds to level of dietary sulfate as explained in Table 2, p. 28.

[b]Values represent averages of 8 values obtained from 4 rats ± standard error of the mean.

[c]Approximately 55% average decrease in conjugation for all diets.

[d]Approximately 85% average decrease in conjugation for all diets.

The data obtained from this experiment (Table 5) show a decrease approaching statistical significance in the tissue coenzyme A level when the rats fed 0.0002% $SO_4^{=}$ + 0.4% cysteine ($0.1>P>0.05$) and 0.42% $SO_4^{=}$ ($0.1>P>0.05$) diets are compared to rats fed 0.1% $SO_4^{=}$ + 0.4% cysteine and 0.42% $SO_4^{=}$ + 0.4% cysteine diets respectively. This decrease in coenzyme A level corresponds to the increase in G:T ratio observed when coenzyme A was omitted from the reaction mixtures. A statistically significant ($P<0.01$) increase in the coenzyme A level was observed when the rats were fed 0.1% $SO_4^{=}$ + 0.4% cysteine as compared with 0.42% $SO_4^{=}$; an increase in coenzyme A was also observed in rats fed 0.42% $SO_4^{=}$ + 0.4% cysteine diet as compared with rats fed 0.0002% $SO_4^{=}$ + 0.4% cysteine diet ($P<0.02$).

Experiment 3

The data presented earlier from experiment 1 have shown that when both taurine and glycine are available in the reaction mixture, taurine is the preferred substrate for conjugation. Literature reports (5, 24) indicate that there are two enzymes, one for glycine and another for taurine conjugation. If so, then taurine should not be the preferred substrate when both glycine and taurine are present in the incubation mixtures in equal concentration. In an attempt to resolve this discrepancy, the kinetics of the enzymes for conjugation were determined. In the first experiment, reaction rate of the enzyme catalyzed reaction was studied in order to determine the time of first order reaction, i.e., time when there was a linear relationship between the time of incubation and formation of bile acid conjugates. These data show (Figure 5) that

TABLE 5

EFFECT OF DIFFERENT LEVELS OF DIETARY SULFATE ON THE LEVEL OF FREE COENZYME A IN MITOCHONDRIAL FREE HOMOGENATE OF RAT LIVER

Diet Number	Level of Dietary $SO_4^=$	Level of Organic S as $SO_4^=$ in Diet	Level of Total S as $SO_4^=$ in Diet	Free Coenzyme A mg/g Fresh Liver
	%			
1	0.0002	0.57	0.57	3.06±0.14[a]
2	0.10	0.57	0.67	3.24±0.12
3	0.42	0.25	0.67	3.08±0.12
4	0.42	0.57	0.99	3.56±0.24

Statistical Comparisons[b]

1 to 2	$0.1 > P > 0.05$
1 to 3	N.S.
1 to 4	$P < 0.02$
2 to 3	$P < 0.01$
2 to 4	N.S.
3 to 4	$0.1 > P > 0.05$

[a]Values represent averages of 10 values obtained from 5 rats ± standard error of the mean.

[b]By paired comparisons (77).

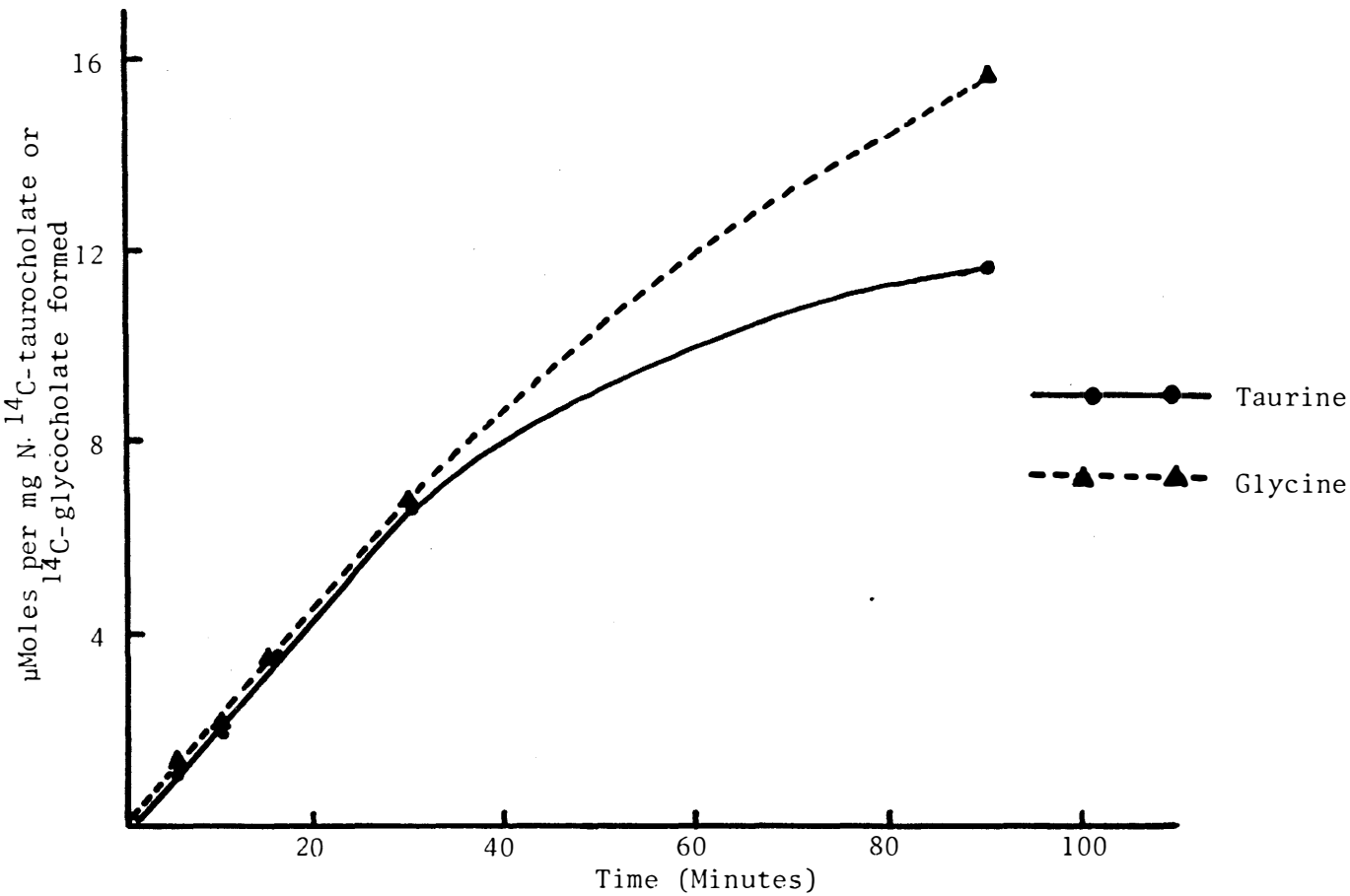

Figure 5. Relationship of ^{14}C-cholic acid conjugation to time of incubation when either taurine or glycine is present in the incubation mixture.

at least up to 15 minutes of incubation, there was a linear relationship between time and glycocholate or taurocholate formation. Therefore, an incubation time of 15 minutes was selected for determination of the Michaelis constant (Km) with either glycine or taurine as substrate. Michaelis constants of enzyme bile acyl transferase for glycine and taurine were determined using Lineweaver and Burk (78) plot, in which reciprocal of the velocity of enzyme catalyzed reaction was plotted against the reciprocal of substrate concentration. These plots demonstrate (Figures 6, 7) that the Km for bile acyl transferase is 15 times greater when glycine is the substrate than when taurine is the substrate, i.e., 15 times more glycine would be required as compared with taurine to achieve the half maximal velocity of the enzyme catalyzed reaction. Competitive inhibition of glycine conjugation by taurine was shown in the next experiment by addition to the reaction mixture of 6.6×10^{-7} M taurine and increasing glycine concentration (about 300 times). At this high concentration of glycine the inhibition caused by the addition of taurine was overcome (Figure 8) and there was an increase in Km for glycine from 3.3×10^{-3} to 2.5×10^{-2} M (Table 6), but there was no change in the intercept 1/V. To further demonstrate the nonspecificity of enzyme bile acyl transferase, β-alanine which is a structural analog of taurine was used to see if β-alanine could also conjugate with cholic acid. So in this experiment, the reaction mixture contained all of the ingredients plus β-alanine. These data show (Table 7) that bile acyl transferase catalyzed the conjugation of cholic acid with β-alanine also, although not as actively as with glycine or taurine. Addition of taurine to the reaction mixture

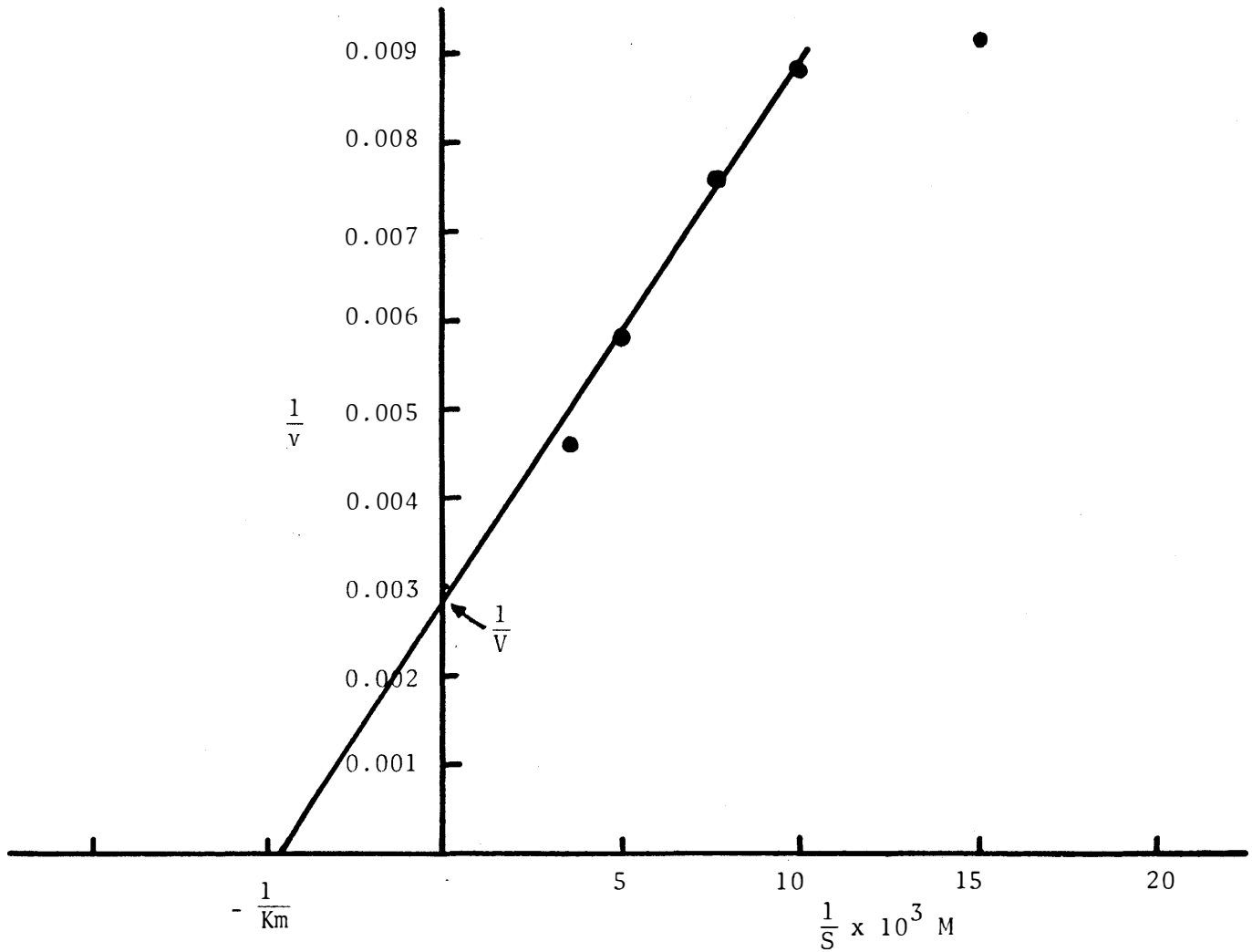

Figure 6. Lineweaver and Burk plot for Michaelis constant (Km) of the enzyme bile acyl transferase when taurine is substrate.

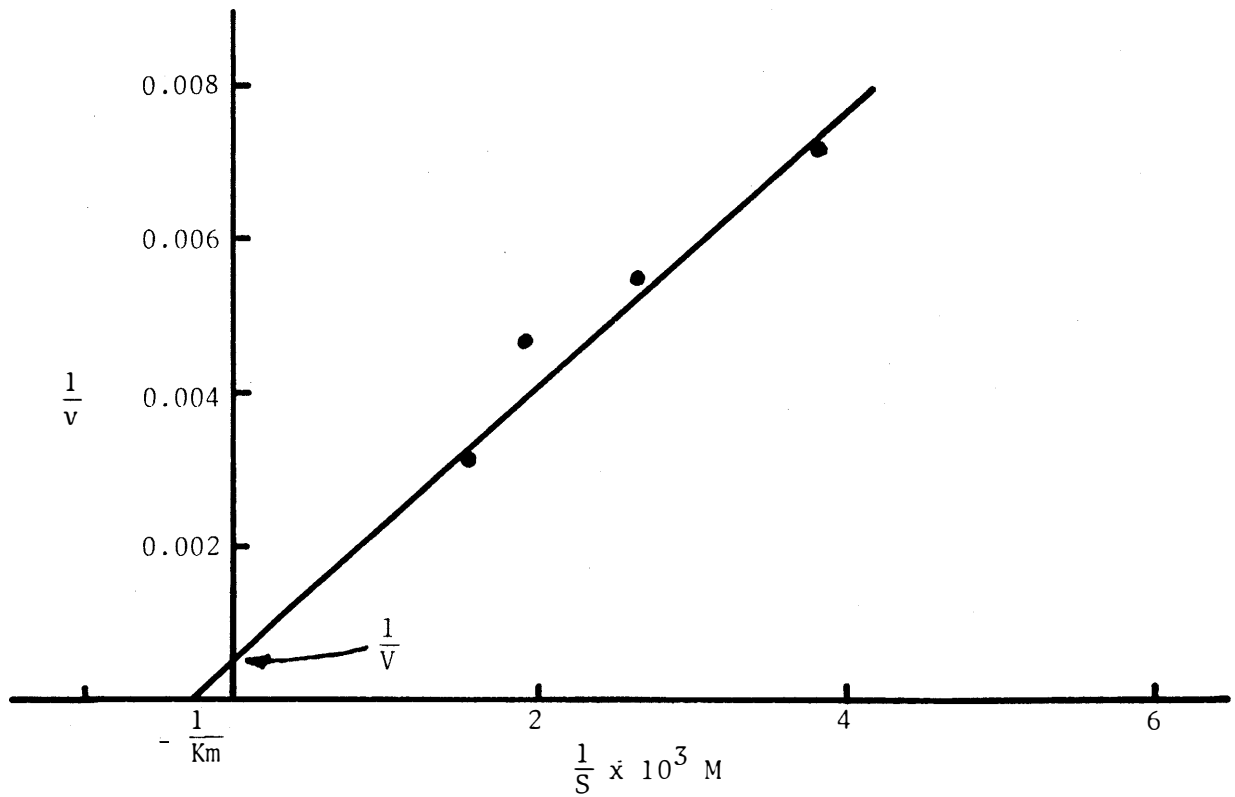

Figure 7. Lineweaver and Burk plot for Michaelis constant (Km) of the enzyme bile acyl transferase when glycine is substrate.

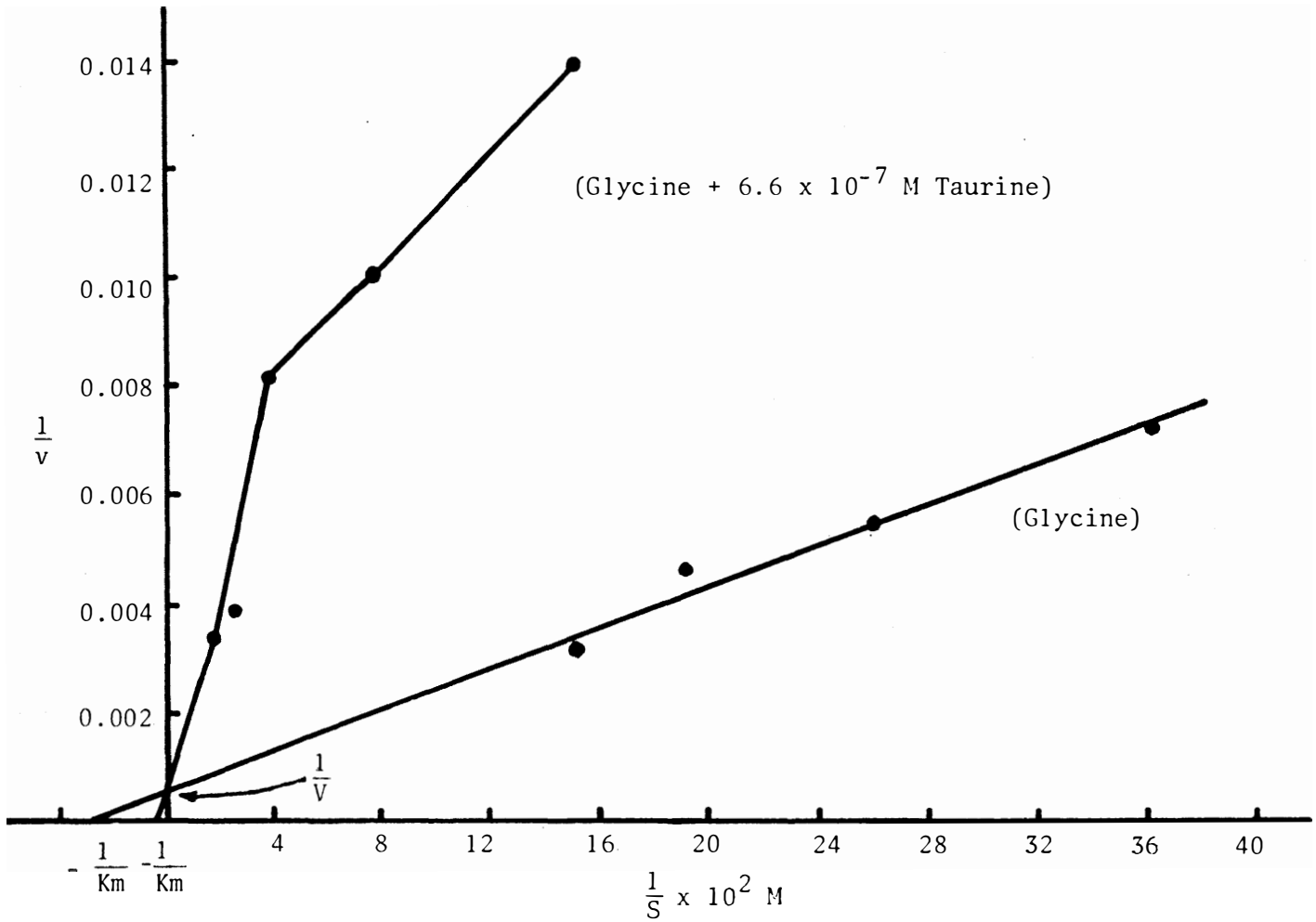

Figure 8. Competitive inhibition of bile acyl transferase by taurine when glycine is substrate.

TABLE 6

MICHAELIS CONSTANTS (Km) OF THE ENZYME BILE ACYL TRANSFERASE FOR GLYCINE AND TAURINE

Substrate	Km (M)
Taurine	2.2×10^{-4}
Glycine	3.3×10^{-3}
Glycine + 6.6×10^{-7} M Taurine	2.5×10^{-2} M

TABLE 7

EFFECT OF DIFFERENT SUBSTRATES ON ^{14}C-CHOLIC ACID CONJUGATION

	μMoles per mg nitrogen		
Substrate	^{14}C-Taurine Conjugate Formed	^{14}C-Glycine Conjugate Formed	^{14}C- β-alanine Conjugate Formed
Taurine	10.64	--	--
Glycine	--	15.04	--
β-alanine	--	--	6.75
Taurine + Glycine	15.02	1.34	--
Taurine + β-alanine	15.85	--	0.66
Glycine + β-alanine	--	15.04	4.72

containing β-alanine completely inhibited this conjugation while addition of glycine also inhibited conjugation with β-alanine but not as much as adding taurine.

CHAPTER V

DISCUSSION

The present investigation tended to supply a missing link observed in previous investigations from this laboratory. Whittle and Smith (1) observed that when the rats were fed the lowest level (0.0002%) of inorganic sulfate, they had an increase in taurine excretion indicating that tissue saturation with taurine had been achieved and an excess of it was being excreted. Since other investigations (22) have shown that taurine conjugation may be a function of taurine availability, the excess of taurine should be utilized for conjugation with bile acids and there should be the lowest G:T ratio in the intestine of rats fed this diet. However, contrary to this speculation, rats fed the diet containing 0.0002% of sulfate had a higher G:T ratio than those fed the diet containing 0.1% of sulfate (2). These observations suggested that factors other than taurine availability must be controlling bile acid conjugation in rats fed these semi-purified diets. Data obtained in this investigation (Table 3, p. 42) appeared to confirm these observations. When the complete incubation mixture was used, little dietary effect was observed on the G:T ratio catalyzed by microsomal preparations.

Bremer (5) demonstrated that coenzyme A was mandatory for *in vitro* conjugation of glycine and taurine with cholic acid. Therefore, since literature reports (63, 64) have indicated that the sulfur status of the animal may affect the tissue level of coenzyme A,

coenzyme A was omitted from the reaction mixture to determine if coenzyme A omission would affect the G:T ratio produced by liver microsomal preparations of rats. When coenzyme A was omitted from the reaction mixture, the G:T ratio was increased in those samples containing microsomal preparations of rats fed diets deficient in either sulfate or cysteine (Table 3, p. 42). These data and consideration of the literature on the synthesis of coenzyme A suggest that the effect of dietary sulfate in altering the G:T ratio might be mediated through the level of coenzyme A in the tissue. The data which are shown in Table 5, p. 46, demonstrate that these diets affect the level of free coenzyme A in the liver; i.e., diets low in sulfate or cysteine cause a deficiency of coenzyme A in the tissue, presumably limiting the availability of coenzyme A for the conjugation of bile acids. Other data (Table 4, p. 44) show that the omission of coenzyme A from the reaction mixture caused an approximate 85% decrease in taurocholate synthesis whereas the decrease in glycocholate synthesis was approximately 55%. These data indicated that the synthesis of taurocholate is more dependent upon the availability of coenzyme A than the synthesis of glycocholate. The combined data indicate that even if there is more taurine in the body, it may not be utilized efficiently for conjugation because of a deficiency of coenzyme A. This interrelationship offers an explanation for the observations in the previous investigations where more taurine was excreted and higher G:T ratios were observed in the rats fed diets low in sulfate.

A number of reports (6, 7) from the literature indicated that taurine was preferred for conjugation in a reaction mixture containing

equal concentrations of glycine and taurine. And it is also reported in the literature (5, 24) that there are two enzymes, taurine bile acyl transferase I for taurine conjugation and glycine bile acyl transferase II for glycine conjugation. If there are two enzymes, then the competition between glycine and taurine for an active site on the enzyme and the preference of taurine for conjugation become difficult to rationalize. In order to resolve this controversy, the Michaelis constants (Km) of the enzyme for glycine and taurine, and glycine in the presence of small amounts of taurine were determined. The results of this experiment indicated that the Km of the enzyme bile acyl transferase is 15 times higher when glycine is the substrate than when taurine is the substrate (Table 6, p. 52). These data allow an explanation of the observed preference for taurine conjugation by the rat liver system. This study further demonstrated that the presence of small amounts of taurine in the incubation mixture inhibits glycine conjugation and that this inhibition may be overcome by increasing the concentration of glycine. These data together with the increased Km for glycine in the presence of taurine and the unchanged $\frac{1}{V}$ indicate that taurine acts as competitive inhibitor of glycine conjugation. The concept of competitive inhibition explains why feeding taurine and not glycine could change the G:T ratio in man (22). The concentration of glycine in the tissue may not rise to a level sufficiently high (approximately 300 times that of taurine) to overcome the inhibition caused by the presence of taurine in the tissue. This investigation explains the reason for difference in bile acid conjugation pattern in different species; for example, the rabbit conjugates only glycine,

not because the enzyme for taurine conjugation is missing as is proposed by Bremer (7) but due to the negligible amount of taurine present in rabbit liver tissue (34). From these studies, it is concluded that liver tissue contains only one bile acyl transferase and that the preferred substrate for this enzyme is taurine due to its lower Km but that this same enzyme may also catalyze acyl transfer of other structurally similar compounds like glycine and β-alanine. The conjugation with β-alanine has also been confirmed by additional experiments and this conjugation is completely inhibited in the presence of taurine but not to the same extent by the presence of glycine. Schersten (24) also speculated about the existence of two bile acyl transferases, but did not do any further studies along these lines, perhaps due to the unavailability of a method to determine the activity of the enzyme bile acyl transferase.

Previous findings from this laboratory (2) indicated that the levels of sulfate and of sulfate and taurine precursors in the diet are important in the dietary management of hypercholesterolemia because they change the G:T ratio. In the present investigation it has been demonstrated that the level of coenzyme A is also crucial in changing the G:T ratio. Therefore, any dietary treatment which alters the level of taurine and/or coenzyme A in the tissue will also change the G:T ratio and may be important in the dietary management of serum cholesterol levels.

CHAPTER VI

SUMMARY

The effect of different levels of dietary sulfate on the relative conjugation of ^{14}C-cholic acid with taurine and glycine in rat liver microsomal preparations was investigated. No diet related differences were observed in the G:T ratio except in rats fed 0.0002% $SO_4^{=}$ + 0.4% cysteine diet. Omission of coenzyme A from the incubation mixture resulted in alterations of the G:T ratio which were related to the level of sulfate in diets. A deficiency of inorganic sulfate or cysteine (0.0002% $SO_4^{=}$ + 0.4% cysteine diet and 0.42% $SO_4^{=}$ diet) caused an increase in the G:T ratio as compared to the G:T ratio observed in rats fed 0.1% $SO_4^{=}$ + 0.4% cysteine diet and 0.42% $SO_4^{=}$ + 0.4% cysteine diet. The overall increase in the G:T ratio in the incubation mixture not containing coenzyme A resulted from a decrease of approximately 85% in taurocholate synthesis and only 55% in glycocholate synthesis. The synthesis of taurocholate was more dependent upon the level of coenzyme A.

Further, there was a decrease in the level of free coenzyme A in the mitochondrial and nuclei free fraction of liver homogenates of rats fed 0.0002% $SO_4^{=}$ + 0.4% cysteine diet and 0.42% $SO_4^{=}$ diet. These are the same diets which caused an increase in the G:T ratio.

Michaelis constant (Km) of the enzyme bile acyl transferase for glycine was 15 times higher than its Km for taurine. The Km for glycine in the presence of taurine was further increased, thereby showing that taurine acts as a competitive inhibitor of glycine conjugation.

These data have demonstrated that any dietary treatment which alters the level of taurine and/or coenzyme A in the tissue will also change the G:T ratio and may be important in the dietary management of serum cholesterol levels.

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