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I am submitting herewith a dissertation written by Jane Vicknair White entitled "Effect of Sulfur Nutrition on Propionyl CoA Carboxylase Activity 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 Nutrition.

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EFFECT OF SULFUR NUTRITION ON PROPIONYL CoA CARBOXYLASE ACTIVITY IN RAT LIVER

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee

Jane Vicknair White

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ABSTRACT

The effect of the level of dietary inorganic sulfate upon the activity of rat liver propionyl CoA carboxylase was investigated. Adult male rats were fed 15% casein diets in which the level of inorganic sulfate was varied from 0.0002% to 0.42%. Propionyl CoA carboxylase activity was determined by measuring the rate of $H^{14}CO_3^-$ fixation in the presence of propionyl CoA. Significant increases in enzyme activity were observed when inorganic sulfate was fed at levels above (0.10% and 0.42%) and below (0.0002%) the established dietary optimum of 0.02%. Thus, attempts were made to determine the mechanism by which inorganic sulfate exerted this effect.

The substitution of propionate or propionate plus CoA for propionyl CoA resulted in a decrease in the rate of the reaction but the pattern of increased enzymatic activity observed when inorganic sulfate was fed at levels above or below the optimum remained unchanged. The omission of reduced glutathione from the reaction mixture produced a similar effect. Thus, the mechanism of the inorganic sulfate effect could not be explained by an effect of inorganic sulfate upon tissue levels of CoA, the acylation of propionate or tissue levels of reduced glutathione.

Mitochondrial-cytoplasmic combinations from rats fed different levels of inorganic sulfate were used to determine whether or not the level of dietary inorganic sulfate affected the availability of a cytoplasmic factor necessary for enzyme synthesis. A significant increase in enzymatic activity was observed when the cytoplasm of rats fed

0.0002% of inorganic sulfate was combined with the mitochondria from rats fed 0.02% of inorganic sulfate when compared to the mitochondrial-cytoplasmic combination from rats fed 0.02% of inorganic sulfate; while the cytoplasm from rats fed 0.02% of inorganic sulfate when combined with the mitochondria from rats fed 0.0002% of inorganic sulfate produced a significant decrease in enzyme activity when compared to the mitochondrial-cytoplasmic combination from rats fed 0.0002% of inorganic sulfate. The addition of biotin to the reaction mixture completely reversed this pattern of activation. Therefore, very low levels of dietary inorganic sulfate appeared to increase the availability of biotin for propionyl CoA carboxylase synthesis.

Increasing amounts of inorganic sulfate were added to the reaction mixture in an attempt to determine the mechanism of the sulfate effect when inorganic sulfate was fed at levels above the established dietary optimum. The addition of inorganic sulfate produced a gradual and eventually significant increase in enzyme activity. The addition of biotin to the reaction mixture caused a 63% decrease in the amount of inorganic sulfate required to produce a significant increase in enzyme activity. Kinetic studies of the enzyme protein indicated that inorganic sulfate is an allosteric activator of propionyl CoA carboxylase.

Increases in enzyme activity at levels of inorganic sulfate supplementation above and below the established dietary optimum were readily explained since very low levels of dietary inorganic sulfate have been shown to increase the methionine requirement while high levels of dietary inorganic sulfate apparently increased the rate of gluconeogenesis. In either case, a catabolism of amino acids is involved and

the carbon skeletons of certain aliphatic amino acids are metabolized via a conversion to propionate.

The data presented in this investigation, therefore, strengthen the concept of inorganic sulfate as a metabolic regulator and demonstrate that the mechanism of the effect of dietary inorganic sulfate upon the activity of propionyl CoA carboxylase is related to an effect of the level of dietary inorganic sulfate upon biotin metabolism.

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CHAPTER I

INTRODUCTION

The nutritional importance of the sulfur containing amino acids is well documented. Investigations in this laboratory, however have established the need to include 0.02% of inorganic sulfate in the diet of a rat if metabolic alterations are to be avoided.

Metabolic alterations occur in rats fed inorganic sulfate at levels above or below the established optimal dietary level of 0.02%. Inorganic sulfate, fed at levels above the optimum (0.10% or 0.42%) caused an increase in liver glycogen and blood glucose levels, an increase in the activity of phosphoenolpyruvate carboxykinase and a stimulation of pyruvate carboxylase. A decrease in the liver level of coenzyme A occurred in rats fed very low levels of inorganic sulfate (0.0002%). Thus, the level of inorganic sulfate in the diet influences both carbohydrate and lipid metabolism in the rat. Such an influence is perhaps mediated through the effect of dietary inorganic sulfate upon tissue levels of coenzyme A.

Propionyl CoA carboxylase (propionyl CoA:ligase (ADP), E.C.6.4.1.3) was chosen as the system in which to examine whether or not inorganic sulfate influences carbohydrate and lipid metabolism through its effect upon tissue levels of CoA since the enzyme is closely related to carbohydrate metabolism and is one whose activity is dependent upon the presence of a short chain acyl derivative of coenzyme A. Whether or not dietary inorganic sulfate

exerts an influence upon the activity of this enzyme will be determined and attempts will be made to elucidate possible mechanisms involved in the production of a diet related effect.

CHAPTER II

REVIEW OF LITERATURE

I. INORGANIC SULFATE

Background

Previous investigations, conducted in this laboratory, have demonstrated that metabolic alterations occur in rats fed diets in which the level of inorganic sulfate is very low (0.0002%). The metabolic alterations observed are a decreased uptake of $^{35}SO_4^{=}$ by rib cartilage mucopolysaccharides(1), an enhanced effect of avitaminosis E[1], an increased excretion of taurine (2), an increase in the glycocholic: taurocholic acid ratio in the small intestine (3), a decreased breaking strength of the aorta (4) and an apparent increase in the requirement for the sulfur-containing amino acids (1).

In addition to the above documentation of the metabolic alterations that occur in rats due to insufficient dietary inorganic sulfate, several positive aspects of dietary inorganic sulfate supplementation have been recorded. Wellers et al. (5) demonstrated that dietary inorganic sulfate supplied all of the sulfur needs of the adult rat except those of protein synthesis; thus the requirement for the sulfur-containing amino acids was decreased. Button et al. (6) demonstrated that dietary inorganic sulfate is well utilized in the formation of mucopolysaccharides. Roe (7) showed that dietary inorganic sulfate

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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.

could be used effectively in the detoxication of indoles.

Such data clearly established the need to include inorganic sulfate in the diet of the rat if metabolic stress was to be avoided; although the exact level of inorganic sulfate supplementation to employ was undefined.

Optimal Dietary Level

The optimal dietary level of inorganic sulfate for the rat, 0.02%, was established by a series of experiments designed to reveal the level of dietary inorganic sulfate at which methionine sparing is maximal (8). When rats were fed diets which contained levels of inorganic sulfate above or below 0.02%, there was an apparent increase in the metabolism of 1-^{14}C-methionine as evidenced by an increased expiration of $^{14}CO_2$. When the same experiment was repeated but test meals of U-^{14}C-cysteine were fed, there was a decrease in the expiration of $^{14}CO_2$ when inorganic sulfate was fed at levels up to and including 0.42% of the diet. Such data were difficult to correlate since any sparing of cysteine should have been reflected as a sparing of methionine. Thus maximal methionine sparing should have occurred at the 0.42% level of dietary inorganic sulfate supplementation. Since no additional effect upon methionine metabolism, above that observed at the 0.02% level of dietary inorganic sulfate supplementation, was evident when this higher level of dietary inorganic sulfate was fed, it was postulated that perhaps higher levels of dietary inorganic sulfate influenced the storage of the carbon skeleton of cysteine. (8) If this were true, the net effect would be a decreased $^{14}CO_2$ expiration; but no additional effect upon methionine metabolism would be

evident because no true sparing of cysteine would have occurred.

An increased storage of the carbon skeleton of cysteine, as liver glycogen, was observed when the amount of ^{14}C recovered as liver glycogen following a test meal of U-^{14}C-cysteine was determined in rats fed inorganic sulfate at levels above 0.02% of the diet.[2] This finding suggests, therefore, that inorganic sulfate plays a role in metabolism in addition to that of methionine sparing.

Metabolic Role

Inorganic sulfate, fed at levels above the optimum (0.10% or 0.42%) causes an increase in liver glycogen[2] and blood glucose levels (3) and an increase in the activity of several key enzymes of carbohydrate metabolism. Inorganic sulfate has been shown to activate phosphoenol-pyruvate carboxykinase and to stimulate pyruvate carboxylase.[3] Low levels of dietary inorganic sulfate (0.0002%) or of dietary cysteine (0.060%) cause a decrease in liver levels of coenzyme A in the rat.[4] Thus the level of inorganic sulfate in the diet influences both the glycogenic flux and lipid metabolism.

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[2]Smith, J. T. (1971) Unpublished data.

[3]Smith, J. T. (1974) Unpublished data.

[4]Verma, R. S. (1974) An investigation of the effect of dietary sulfate on bile acid conjugation and kinetic studies of bile acyl transferase in rat liver microsomes. Unpublished Doctoral Dissertation, The University of Tennessee, Knoxville.

II. PROPIONYL CoA CARBOXYLASE

Reaction Catalyzed

Propionyl CoA carboxylase (propionyl CoA:ligase (ADP) E.C.6.4.1.3.) catalyzes the biotin-dependent carboxylation of propionyl CoA to form methylmalonyl CoA (9) by the reaction shown in Figure 1. This enzyme participates not only in the oxidation of propionic acid but also in the oxidation of compounds that are converted to propionate within the animal body.

Sources of Propionate

Propionate is the product of several metabolic reactions. In mammals, propionate arises from the β-oxidation of the odd chain fatty acids (10-12); the degradation of the aliphatic amino acids valine (13-14), isoleucine (14), methionine and threonine (15); the catabolism of the pyrimidine, thymine (15); and the oxidation of phytanic acid (16).

Historical Background

The study of propionate metabolism in animal tissues began in 1912 when Ringer (17) observed the complete conversion of propionate to glucose in diabetic dogs. Deuel et al. (10) corroborated this finding by demonstrating a substantial increase in the liver glycogen content of normal rats fed test meals of sodium propionate. Although the conversion of propionate to glucose or glycogen could be readily demonstrated, the nature of the intermediary reactions was obscure.

$$ATP + HCO_3^- + \text{propionyl CoA} \xrightleftharpoons{Mg^{++},\ \text{biotin}} ADP + Pi + \text{methylmalonyl CoA}$$

Figure 1. Reaction catalyzed by propionyl CoA carboxylase.

Several investigators (18, 19) attributed the glycogenic nature of propionate to its more or less direct conversion to pyruvate via acrylate and lactate. However, studies involving the use of isotopic propionate did not support this theory. Although the observed isotopic distribution patterns in the pyruvate formed were similar, when the in vivo conversions of identically labeled propionate and lactate were compared, there were basic differences between the two patterns which indicated that the "direct" conversion of propionate to pyruvate was improbable. Instead, it was postulated that the conversion of propionate to pyruvate occurred via a symmetrical compound such as succinate (20-22). Such a conversion would explain the isotopic data as well as the glycogenic properties of this compound.

The theory that propionate is converted to the dicarboxylic acids before being converted to pyruvate was supported by the findings of Lardy and Peanasky (23); that extracts of liver mitochondria from biotin sufficient rats, when supplemented with Mg^{++} or Mn^{++} and adenosine triphosphate (ATP) catalyzed the conversion of propionate to succinate by CO_2 fixation. In extracts of liver mitochondria from biotin deficient rats, the rate of propionate carboxylation was greatly reduced. Biotin was, therefore, implicated in propionate metabolism.

Additional requirements were described for the conversion of propionate to succinate by crude mitochondrial extracts of rat liver (24) and pig heart (25). The optimal ratio of ATP to Mg^{++} or Mn^{++} in such preparations is 1:1. When the concentration of ATP was increased above that of the divalent cation, the reaction was progressively

inhibited; while increases in the metal ion concentration reversed this inhibition (24, 25). An absolute requirement for CoA was demonstrated (24, 25) and synthetic propionyl CoA was a much more effective substrate than propionate + CoA (25). However, substitution of propionyl CoA for propionate + CoA in the reaction mixture did not decrease the requirement for ATP. Thus, an energy requirement was demonstrated for the carboxylation process beyond that which was required for substrate activation (24, 25).

Katz and Chaikoff (26), while studying the metabolism of propionate in rat liver slices, identified methylmalonate rather than succinate as the immediate product of propionate carboxylation. Flavin et al. (27) suggested an activated form of methylmalonate, methylmalonyl CoA, as the immediate product of propionyl CoA carboxylation and showed that under optimal conditions for the carboxylation of propionate by pig heart preparations, little succinate was formed. Flavin and Ochoa (25) identified methylmalonyl CoA as the immediate product of propionyl CoA carboxylation.

Purification (28) of the propionyl CoA carboxylation system of pig heart mitochondrial extracts revealed that the conversion of propionyl CoA, CO_2 and ATP to methylmalonyl CoA, adenosine diphosphate (ADP) and orthophosphate is catalyzed by a single enzyme, propionyl CoA carboxylase, which binds both CO_2 and propionyl CoA before carboxylation occurs.

Distribution

Propionyl CoA carboxylase is present in mitochondrial extracts of pig heart (25) and of liver from beef, guinea pig, hog, rabbit, sheep, and rat; however, almost no activity was observed in mitochondrial preparations from rat brain, heart, kidney, lung, skeletal muscle, spleen, or tongue (29).

Scholte (30) in a study of the intracellular and intramitochondrial distribution of rat liver propionyl CoA carboxylase found that 75% of the enzymic activity was localized in the mitochondria. A comparison of the intramitochondrial distribution of propionyl CoA, with that of marker enzymes, revealed that the enzyme is very loosely bound to the inner membrane or is free.

Molecular Weight and Related Properties

Propionyl CoA carboxylase has been crystallized and extensively characterized from mitochondrial extracts of pig heart preparations (31-24). The enzyme is homogeneous throughout electrophoresis and ultracentrifugation and has an isoelectric point of pH 6.1. The molecular weight of propionyl CoA carboxylase, based on the diffusion constant (2.58×10^{-7}), sedimentation constant (19.7 S) and viscosity determination ($[\eta]=0.083$ deciliters per g), is 700,000. The enzyme contains 1 mole of bound biotin per 175,000 g of enzyme or 4 moles per mole of enzyme. Thus propionyl CoA carboxylase is thought to consist of 4 subunits, each with a molecular weight of 175,000 and each containing 1 mole of bound biotin. On ultracentrifugation with 7M urea,

the enzyme disassociates into inactive subunits of 2.5 S. The molecular weight and biotin content of these subunits has not been determined.

Substrate Specificity

The apparent substrate affinity constants for the enzyme isolated from pig heart mitochondria have been determined. Apparent Km values for ATP, HCO_3^- and propionyl CoA are calculated to be 8×10^{-5}, 2.5×10^{-3}, and 2.7×10^{-4}M respectively. (9, 28)

Tietz and Ochoa (28) showed that the nucleoside -5- triphosphate specificity of pig heart propionyl CoA carboxylase is restricted to ATP. Neither guanosine triphosphate (GTP), uridine triphosphate (UTP), cytidine triphosphate (CTP), or inosine triphosphate (ITP) could replace ATP.

Although maximally active with propionyl CoA, the enzyme has been shown to carboxylate a variety of acyl CoA esters including butyryl CoA, crotonyl CoA and acetyl CoA. The relative rates of carboxylation of these substrates when the rate of propionyl CoA carboxylation is assumed to be 100 are 6, 3 and 1 respectively (32).

pH Optimum

The optimum pH of the reaction lies between pH 8.0 and 8.5. Sharp decreases in enzyme activity occur on either side of the optimum. (32)

Enzyme Activation or Inhibition

As stated previously, both Mg^{++} and biotin are required for enzyme activity (9). Since propionyl CoA carboxylase is a biotin

containing enzyme, it is inhibited by stoichiometric amounts of avidin. The prior administration of excess d-biotin will prevent this inhibition. (35)

The monovalent cations Cs^+, Rb^+, NH_4^+, and K^+ have been shown to increase the activity of rat liver and pig heart propionyl CoA carboxylase; however, the degree of stimulation shown with crystalline enzyme preparations was lower than that shown in crude enzyme preparations (36). Of the monovalent cations shown to cause an activation of propionyl CoA carboxylase, the effect of K^+ has been most intensely investigated. Although an absolute requirement for K^+ could not be demonstrated, Edwards and Keech (37) showed that the addition of K^+ ions to the reaction mixture caused a significant decrease in the apparent Km value for bicarbonate. The apparent Km values for ATP and propionyl CoA remained unchanged. Potassium ions caused an increased rate of binding of N-^{14}C-ethylmaleimide to the enzyme protein and increased the loss of enzyme activity caused by this compound. These investigators concluded, therefore, that K^+ induced a conformational change in the enzyme protein at the HCO_3^- binding site.

Although a requirement for reduced glutathione could not be demonstrated in crude enzyme preparations (24), the addition of reduced glutathione to the highly purified enzyme system resulted in a marked increase in enzyme activity (28). This finding suggested that propionyl CoA carboxylase is dependent upon the integrity of sulfhydryl groups. Kaziro et al. (36) demonstrated that the inhibitory effects of the sulfhydryl binding reagents parachloromercuribenzoate (pCMB) and

iodoacetamide could be reversed by the addition of reduced glutathione to the reaction mixture. Edwards and Keech (38), while investigating the function of the essential thiol group of propionyl CoA carboxylase, demonstrated that a cysteine residue is involved in the reaction sequence. When N-ethylmaleimide, a compound which irreversibly alkylates cysteine residues, was added to the reaction mixture, the binding of propionyl CoA to the enzyme protein was decreased while the binding of ATP and HCO_3^- remained the same. It was concluded, therefore, that a thiol group is involved in the reaction sequence in the formation of the enzyme-propionyl CoA complex.

Mechanism of Action

The ATP and Mg^{++} dependent carboxylation of propionyl CoA to form methylmalonyl CoA is a readily reversible reaction. Reversibility was demonstrated by observing the net formation of labeled ATP from ADP and ^{32}P-orthophosphate and from ^{14}C-ADP and orthophosphate in the presence of methymalonyl CoA (9). The average value of the apparent equilibrium constant for propionyl CoA carboxylase is K' = 5.7 at pH 8.1 and 28°. This corresponds to a free energy change (ΔF_{301}) of -1028 calories per mole and is further proof that the reaction is readily reversible with the equilibrium position slightly favoring the carboxylation of propionyl CoA. (31)

Kaziro and Ochoa (39) demonstrated that the overall reaction catalyzed by propionyl CoA carboxylase is the result of the two partial, reversible reactions shown in Figure 2. The occurrence of the first partial, reversible reaction was indicated by the ability of the enzyme

(a) $ATP + HCO_3^- + \text{biotin-enzyme} \xrightleftharpoons{Mg^{++}} \text{carboxybiotin-enzyme} + ADP + Pi$

(b) $\text{carboxybiotin-enzyme} + \text{propionyl CoA} \rightleftharpoons \text{methylmalonyl CoA} + \text{biotin-enzyme}$

Sum: $ATP + HCO_3^- + \text{propionyl CoA} \xrightleftharpoons{Mg^{++},\ \text{biotin}} ADP + Pi + \text{methylmalonyl CoA}$

Figure 2. Propionyl CoA carboxylase reaction sequence.

to catalyze an ATP-^{32}Pi and ATP-^{32}P- labeled ADP exchange dependent upon all of the components of reaction (a) but independent of the components of reaction (b). The occurrence of the second partial, reversible reaction was demonstrated by the ability of propionyl CoA carboxylase to catalyze a quantitative transfer of $^{14}CO_2$ from the $CO_2\sim$ biotin-enzyme complex to propionyl CoA to form methylmalonyl CoA and to catalyze the decarboxylation of ^{14}C-methylmalonyl CoA to form propionyl CoA and the CO_2-biotin-enzyme complex dependent upon the components of reaction (b) but independent of the components of reaction (a).

Substrate quantities of the $CO_2\sim$biotin-enzyme complex were isolated following a short incubation of propionyl CoA carboxylase with ATP, Mg^{++} and HCO_3^-. The decarboxylation of this complex in the presence of ADP, Pi and Mg^{++}, as well as the transfer of the labile carboxyl group of the complex to propionyl CoA to form methylmalonyl CoA, confirmed the presence of an active $CO_2\sim$biotin-enzyme complex intermediate (34).

The ^{18}O experiments of Kaziro et al. (39) demonstrated that the oxygen for ATP cleavage is derived from bicarbonate since one bicarbonate oxygen atom appears in the released orthophosphate and two appear in the free carboxyl group of methylmalonyl CoA. Bicarbonate, therefore, was proven to be the reactive species of CO_2 in the propionyl CoA carboxylase reaction. The binding of CO_2 to the biotin-enzyme complex has been suggested to occur by the reaction mechanism shown in Figure 3; with a nucleophyllic attack of the biotin nitrogen on the bicarbonate carbon being followed by an attack of the bicarbonate

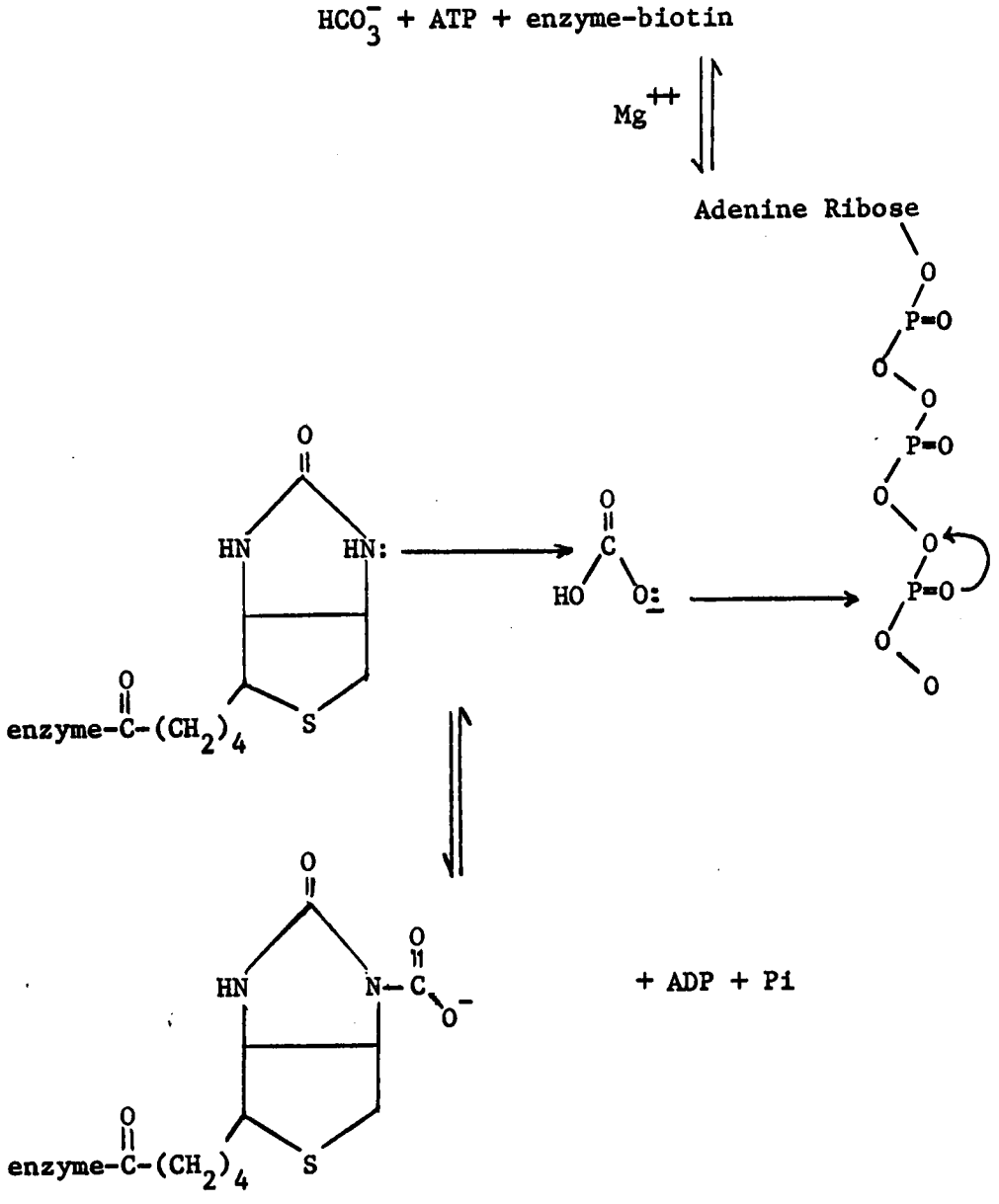

Figure 3. Suggested mechanism for the carboxylation of the enzyme-biotin complex.

oxygen on the terminal phosphate of ATP. (40, 41)

The mechanism of transfer of the carboxyl group of the CO_2~ biotin-enzyme complex to propionyl CoA to form S-methylmalonyl CoA was described in a review by Alberts and Vagelos (9) and is thought to involve simultaneous proton removal and proton transfer. Two-^{3}H-propionyl CoA was synthesized with the isotope in either the 2S or 2R position. These compounds were then used to show that the 2R hydrogen enters the solvent while the 2S hydrogen is retained in the methylmalonyl CoA formed. The rate at which the 2R hydrogen entered the solvent was proportional to the rate of methylmalonyl CoA formation.

Control of Enzyme Synthesis

Kosow and Lane (42) have isolated an enzyme system, from biotin deficient rat liver, capable of forming propionyl CoA holocarboxylase from its apocarboxylase and d-biotin. Alumina Cγ fractionation of the rat liver extracts caused a resolution of the system into two components, one which contained the apocarboxylase and the other which contained an activating enzyme termed holocarboxylase synthetase (43).

Siegel et al. (44, 45) demonstrated that propionyl CoA holocarboxylase synthesis is dependent upon biotin, ATP, Mg^{++} and the presence of both the apocarboxylase and the holocarboxylase synthetase preparations. Biotinyl-AMP (adenosine monophosphate) completely replaced biotin and ATP in the formation of the holocarboxylase which suggested that biotinyl-AMP functions in the final stage of apocarboxylase activation.

The synthesis of propionyl CoA holocarboxylase is believed to proceed by the series of reactions shown in Figure 4, with holocarboxylase synthetase catalyzing both the formation of biotinyl-AMP and its union with the apocarboxylase (45).

Recently Murthy and Mistry[5] reported the synthesis of propionyl CoA holocarboxylase by a mitochondrial preparation of biotin deficient chicken liver and stated that this preparation contained both the apocarboxylase and the holocarboxylase synthetase. These investigators subsequently reported[6] an increased activation of mitochondrial propionyl CoA apocarboxylase by a cytoplasmic factor from the same type of preparation.

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[5]Murthy, P. N. A. & Mistry, S. P. (1973) Synthesis of propionyl CoA holocarboxylase by a partially purified preparation from biotin deficient chicken liver. Fed. Proc. *32*, 950.

[6]Murthy, P. N. A. & Mistry, S. P. (1974) Activation of mitochondrial propionyl CoA apocarboxylase and synthesis of acetyl CoA holocarboxylase by a cytosolic preparation from biotin deficient chicken liver. Fed. Proc. *33*, 706.



Figure 4. Series of reactions leading to the synthesis of propionyl CoA holocarboxylase catalyzed by holocarboxylase synthetase.

CHAPTER III

EXPERIMENTAL PROCEDURE

I. GENERAL PLAN

Based on the literature and experimental evidence cited earlier, this investigation was initiated to determine what effect, if any, the level of dietary inorganic sulfate has upon the activity of rat liver propionyl CoA carboxylase. The liver was chosen as the site for investigation since a study by Friedberg et al. (29) showed that propionyl CoA carboxylase activity was very low or absent in other rat tissues.

Adult male rats of a Wistar, Sprague-Dawley, Long-Evans cross strain served as tissue donors throughout this investigation. The animals were group housed in galvanized steel cages according to diet and were given feed and water ad libitum. The rats were killed by a sharp blow to the head, decapitated, and their livers were removed for enzyme assay. Propionyl CoA carboxylase activity was assessed according to the method of Georgio and Whitaker (46) which is based upon measurement of the rate of $H^{14}CO_3^-$ fixation in the presence of propionyl CoA.

Once a relationship between the level of dietary inorganic sulfate and propionyl CoA carboxylase activity was established, a series of experiments was performed to determine the mechanism by which inorganic sulfate exerted this effect. Experiments were conducted to determine whether the effect of level of dietary inorganic sulfate was manifested through its effect upon 1) tissue levels of CoA, 2) tissue levels of reduced gluthathione, 3) a cytoplasmic factor necessary for

enzyme synthesis and/or 4) the enzyme protein itself.

II. METHODS

Diets

All rats, except those used to study the effect of inorganic sulfate upon the enzyme protein itself, were fed Rat Chow[7] and tap water ad libitum until they were approximately 150-200 g. At this time they were fed distilled water and one of the semi-purified diets shown in Table 1 ad libitum. The rats used to study the effect of inorganic sulfate upon the enzyme protein itself were fed Rat Chow and tap water ad libitum until the day of sacrifice.

The diets shown in Table 1 are a variation of the diets of Caputto et al. (47). The salt mixture is that of Hubbell et al. (48) as modified by Pendergrass.[8] The semi-purified diets were fed for a period of 17 days since Whittle and Smith (2) demonstrated that metabolic alterations, due to the level of inorganic sulfate in the diet, could be detected within a period of 2 weeks when rats were fed the diets shown in Table 1.

Preparation of Enzyme Solutions

Two methods of preparing the enzyme for assay were used. All homogenizations were carried out in an ice bath, using a motor-driven Thomas size C homogenizer with a Teflon pestle.

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[7] Ralston Purina Company, Saint Louis, Missouri 63188. Approximate inorganic sulfate composition equals 0.02%.

[8] Pendergrass, B. J. (1961) The interrelationship of tocopherol and sulfur metabolism. Unpublished Master's Thesis, University of Tennessee, Knoxville.

TABLE 1

COMPOSITION OF SEMI-PURIFIED DIETS

Component	0.0002% $SO_4^=$	0.02% $SO_4^=$	0.10% $SO_4^=$	0.42% $SO_4^=$
		g/100g		
Casein[1]	15.00	15.00	15.00	15.00
Cornstarch	30.00	30.00	30.00	30.00
Sucrose	30.00	30.00	30.00	30.00
Vegetable oil[2]	2.00	2.00	2.00	2.00
Vegetable shortening[3]	6.00	6.00	6.00	6.00
Vitamin mixture[4]	2.00	2.00	2.00	2.00
Non-nutritive bulk[5]	11.66	11.68	11.75	11.50
Basic salt mixture[6]	1.34	1.34	1.34	1.34
DL methionine[1]	0.66	0.62	0.50	0.50
$CaCO_3$	1.34	1.32	1.23	0.91
$CaSO_4 \cdot 2H_2O$	0.00	0.04	0.18	0.75

[1]Nutritional Biochemicals Corporation, Cleveland, Ohio 44128.

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

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

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

[5]Alphacel, Nutritional Biochemicals Corporation, Cleveland, Ohio 44128.

[6]1.34g equals in mg: 92.8 $MgCO_3$, 207.0 NaCl, 336.0 KCl, 636.0 KH_2PO_4, 61.5 $FePO_4 \cdot 2H_2O$, 0.2 KI, 0.3 NaF, 1.8 $AlK(SO_4)_2 \cdot 12H_2O$, 2.1 Cu $(C_2H_3O_2) \cdot H_2O$, 1.2 $MnCl_2 \cdot 6H_2O$.

Liver homogenate. Ten percent liver homogenates were prepared with cold 0.1M Tris -HCl (tris (hydroxymethyl) aminomethane) buffer, pH 7.2. Seventy-five ml of each homogenate were placed in a 100 ml beaker and were sonicated for 90 seconds with a Bronwill Biosonik Sonicator[9] set at maximum power and maximum transducence. The homogenates were sonicated to remove the possibility that the effect of dietary inorganic sulfate upon enzyme activity was due to an effect upon mitochondrial membrane permeability. Scholte (30) also demonstrated that the activity of propionyl CoA carboxylase is greatly increased by sonication. The sonicated preparations were centrifuged at 30,000 x g in a Lourdes Beta-fuge using a 9RA head for 20 minutes at 4° to remove nuclear and mitochondrial remnants. Ten ml of the clear supernatant fluid from each of these preparations were decanted, diluted with an equal volume of 0.1M Tris-HCl buffer, pH 7.2, and mixed by inversion. Similar enzyme preparations were used in all studies except those in which the effect of dietary inorganic sulfate upon a cytoplasmic factor necessary for enzyme synthesis was investigated.

Tissue fractions. Twenty percent liver homogenates were prepared with cold 0.1M Tris-HCl buffer, pH 7.2, and were centrifuged at 600 x g in a Lourdes Beta-fuge using a 9RA head for 10 minutes at 4° to remove the nuclei. The resultant supernatant fluid was decanted and saved and the nuclei were discarded. Mitochondria were isolated by centrifuging the supernatant fluid at 30,000 x g for 10 minutes at 4°. The supernatant fluid, which will be referred to as the cytoplasm, was decanted

[9]Bronwill Scientific, Rochester, New York 14603.

and saved. The mitochondria were washed once with 0.1M Tris-HCl buffer, pH 7.2, and were centrifuged at 30,000 x g for 10 minutes at 4°. At the end of this period, the buffer solution in which the mitochondria were washed was decanted and discarded. A Thomas size A homogenizer with a Teflon pestle rotated gently by hand was used to resuspend the mitochondria in 15 ml of 0.1M Tris-HCl buffer, pH 7.2. Two ml portions of the resuspended mitochondria and of the cytoplasm were recombined for enzyme assay as shown in Table 2. Preliminary experiments with the mitochondrial-cytoplasmic combinations shown in Table 2 ruled out any effect of dietary inorganic sulfate on a cytoplasmic factor necessary for enzyme synthesis at the 0.10% and 0.42% level of dietary inorganic sulfate supplementation. Further investigation was restricted, therefore, to the mitochondrial-cytoplasmic combinations from rats fed 0.0002% and 0.02% of inorganic sulfate as shown in Table 3.

Enzyme Assays

The same general procedure was used to determine propionyl CoA carboxylase activity, although the dietary treatment, certain constituents of the reaction mixture, and/or the preparation of the enzyme solutions were varied depending upon the particular inorganic sulfate effect being investigated.

Determination of liver propionyl CoA carboxylase activity from rats fed different levels of inorganic sulfate in the diet. The activity of propionyl CoA carboxylase was determined according to the method of Georgio and Whitaker (46). Unless specifically stated otherwise, each reaction tube contained in a final volume of 0.75 ml the constituents

TABLE 2

RAT LIVER MITOCHONDRIAL-CYTOPLASMIC COMBINATIONS USED TO SURVEY THE EFFECT OF LEVEL OF DIETARY INORGANIC SULFATE ON A CYTOPLASMIC FACTOR NECESSARY FOR ENZYME SYNTHESIS

Liver mitochondria		Liver cytoplasm
rats fed % of dietary SO_4	combined with	rats fed % of dietary SO_4
0.0002		0.42
0.02		0.0002
0.02		0.02
0.02		0.42
0.10		0.02
0.10		0.42
0.42		0.02
0.42		0.42

TABLE 3

RAT LIVER MITOCHONDRIAL-CYTOPLASMIC COMBINATIONS USED TO IDENTIFY THE EFFECT OF LEVEL OF DIETARY INORGANIC SULFATE ON A CYTOPLASMIC FACTOR NECESSARY FOR ENZYME SYNTHESIS

Liver mitochondria		Liver cytoplasm
rats fed % of dietary SO_4	combined with	rats fed % of dietary SO_4
0.0002		0.0002
0.0002		0.02
0.02		0.0002
0.02		0.02

listed in Table 4 and 0.1 ml of a 10% liver homogenate prepared as described previously. All assays were run in duplicate and duplicate blanks, without propionyl CoA, were run with each series of assays. Incubations were initiated by enzyme addition and were carried out with air as the gas phase at 37.5° for 10 minutes in an Eberbach water-bath shaker. Incubations were stopped by the addition of 0.1 ml of 50% trichloroacetic acid. The samples were removed from the water-bath, covered, and were allowed to sit at least 24 hours at room temperature. Fifty µl of the resultant supernatant fluid from each sample were spotted on 1 x 4.5 cm strips of Whatman #3 filter paper. The strips were allowed to air dry for at least 30 minutes. Each strip was placed in a liquid scintillation vial with 20 ml of a scintillation cocktail containing 12g PPO[10] (2, 5 diphenyloxazol) per liter of toluene. The radioactivity on the strips was evaluated using a Picker Nuclear Liquimat 220 Liquid Scintillation Spectrometer. The channels ratio method was used to correct for quenching.

Enzyme activity was expressed as nanomoles of substrate converted/mg N calculated from the specific activity of the $H^{14}CO_3^-$ as shown in the following formula:

$$\text{n moles/mg N} = \frac{\text{CPM/mg N}}{\text{CPM/n mole } H^{14}CO_3^-}$$

Measurement of CoA effect. Propionyl CoA carboxylase activity was determined as described previously; however, propionyl CoA (Table 4) was replaced by either 3.1 µ moles of sodium propionate or 3.1 µ moles of

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[10] Packard Instrument Company, Inc., 2200 Warrenville Road, Downers Grove, Illinois 60515.

TABLE 4

COMPONENTS OF THE REACTION MIXTURE USED TO DETERMINE PROPIONYL CoA CARBOXYLASE ACTIVITY

Component	Concentration[1]
	μ moles/tube
Tris-HCl, pH 8.5[2]	60.0
$NaH^{14}CO_3$[3]	9.4
Na_2ATP[2]	2.0
Reduced glutathione[2]	2.5
$MgCl_2$	5.0
KCl	75.0
Propionyl CoA[2]	1.0

[1]Concentration of components is μ moles in 0.75 ml final volume.

[2]Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178.

[3]ICN Pharmaceuticals Incorporated, 272 Campus Drive, Irvine, California 92664. Diluted to give a specific activity of 3.4 to 36.0 x 10^4 cpm/μ M bicarbonate.

sodium propionate plus 1.9 μ moles coenzyme A per tube. These substitutions were made to determine whether the effect of dietary inorganic sulfate upon propionyl CoA carboxylase activity resulted from the effect of dietary inorganic sulfate upon tissue levels of CoA or upon the enzyme responsible for the acylation of propionate.

Measurement of reduced glutathione effect. Since propionyl CoA carboxylase is an enzyme which requires the integrity of sulfhydryl groups for its activity, reduced glutathione was omitted from the reaction mixture (Table 4) and the activity of the enzyme was determined as described previously. Whether the effect of dietary inorganic sulfate upon tissue levels of reduced glutathione was the mechanism by which inorganic sulfate exerted its effect upon propionyl CoA carboxylase activity could then be determined.

Measurement of the effect of dietary inorganic sulfate upon a cytoplasmic factor necessary for enzyme synthesis. The reaction mixture described previously (Table 4) was modified, by incorporating the suggestions of Murthy and Mistry[6], to determine the effect of dietary inorganic sulfate upon a cytoplasmic factor necessary for enzyme synthesis. Each tube contained in a final volume of 0.75 ml the components listed in Table 5 plus 0.1 ml of one of the mitochondrial-cytoplasmic combinations shown in Tables 2 and 3. Each assay was performed with and without the addition of 0.5 μ moles of biotin. The remainder of the assay was conducted as described previously.

Measurement of the effect of inorganic sulfate upon the enzyme protein (kinetics of the propionyl CoA carboxylase reaction). This portion of the investigation was designed to measure the effect of inorganic sulfate upon the kinetics of the propionyl CoA carboxylase reaction and, therefore, upon the enzyme protein itself. Graded amounts of sodium sulfate (1.87, 3.75, 5.62, 7.50, 15.00, 22.50, and 30.00 μ moles/tube) were added to the assay system (Table 4 plus 0.1 ml of a 10% liver homogenate) and enzyme activity was determined as described previously. Each assay was evaluated with and without the addition of 0.5 μ moles of biotin. Based on the data obtained using graded levels of sodium sulfate, a level of sodium sulfate was selected (22.50 μ moles/tube) above which there was no apparent change in enzyme activity, to determine the effect of substrate concentration on propionyl CoA carboxylase activity with and without added inorganic sulfate. Substrate concentrations (2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 x 10^{-5} M) were converted to their logarithmic equivalents and a plot of observed velocity/maximum velocity versus the logarithmic equivalent of the substrate concentration was plotted. (Table 5.)

Determination of the Nitrogen Content of the Enzyme Solutions

The nitrogen content of the enzyme solutions was measured by the micro-Kjeldahl method as standardized by Willets and Ogg (49) for all enzyme preparations tested.

Duplicate volumes of the enzyme preparation to be analyzed (1 ml of the supernatant fluid from the 10% liver homogenate, 0.5 ml of the mitochondrial-cytoplasmic combinations) were pipetted into 30 ml

TABLE 5

COMPONENTS OF THE REACTION MIXTURE USED TO DETERMINE THE EFFECT OF DIETARY INORGANIC SULFATE UPON A CYTOPLASMIC FACTOR NECESSARY FOR ENZYME SYNTHESIS

Component	Concentration
	μ moles/tube
Tris-HCl, pH 7.5[1]	60.0
$NaH^{14}CO_3$[2]	9.4
Na_2ATP[1]	2.0
Reduced glutathione[1]	2.5
$MgCl_2$	5.0
KCl	75.0
$Na_3C_6H_5O_7 \cdot 2H_2O$	5.0
Propionyl CoA[1]	1.0

[1]Sigma Chemicals Company, P.O. Box 14508, St. Louis, Missouri 63178.

[2]ICN Pharmaceuticals Incorporated, 272 Campus Drive, Irvine, California 92664. Diluted to give a specific activity of 9.6×10^4 cpm/μ M bicarbonate.

micro-Kjeldahl flasks which contained 1.25 to 1.35 g of Na_2SO_4 and 35 to 45 mg of HgO. Two ml of concentrated sulfuric acid were added to each flask and the contents were digested on a micro-Kjeldahl heating apparatus until clear (approximately 12 hours). The flasks were cooled, and 5 ml of distilled water were added to each flask to dissolve the white crystalline material which had formed. The contents of the flask were quantitatively transferred to a steam distillation apparatus. A 125 ml Erlenmeyer flask containing 5 ml of a 4% boric acid solution (40g of H_3BO_3 dissolved and diluted to 1000 ml with distilled water) and 4 drops of methyl red-bromocresol green indicator (0.83g bromocresol green and 0.17g methyl red diluted to 500 ml with 95% ethanol) were placed under the condenser so that the tip of the condenser extended into the boric acid solution. Approximately 8 ml of a sodium hydroxide-sodium thiosulfate solution (50g NaOH and 5g $Na_2S_2O_3 \cdot H_2O$ diluted to 100 ml with distilled water) were added to the apparatus and the distillation process began. Approximately 45 ml of distillate were collected. The amount of nitrogen present in the distillate was determined by titration of the distillate with 0.01 N HCl (3.6g constant boiling HCl diluted to 2000 ml with distilled water). Milligrams of nitrogen were calculated by the following formula:

(ml HCl) x (N of acid) x (mEq weight of nitrogen) = mg of nitrogen

Statistics

All data were analyzed according to Duncan's new multiple range test for equal replications as described by Steel and Torrie (50).

CHAPTER IV

RESULTS

Inorganic sulfate, when fed at levels above or below the established dietary optimum of 0.02%, caused a significant increase ($P<0.05$) in the activity of rat liver propionyl CoA carboxylase (Table 6). Attempts were made, therefore, to determine the mechanism by which inorganic sulfate exerted this effect.

I. MEASUREMENT OF CoA EFFECT

Sodium propionate was substituted for propionyl CoA in the reaction mixture, in an attempt to determine whether or not the mechanism by which the level of dietary inorganic sulfate increased the activity of propionyl CoA carboxylase was due to an effect of the level of dietary inorganic sulfate upon tissue levels of CoA. A comparison of the data presented in Table 7 with those in Table 6 shows that the substitution of sodium propionate for propionyl CoA decreased the rate of the reaction but did not alter the pattern of increased enzymatic activity observed when inorganic sulfate was fed at levels above or below the established dietary optimum of 0.02%.

The next experiment was conducted to determine whether or not the level of dietary inorganic sulfate affected the enzyme responsible for the acylation of propionate. Sodium propionate plus CoA were substituted for propionyl CoA in the reaction mixture. The data presented in Table 8 were compared with those presented in Table 6. A decrease of approximately

TABLE 6

EFFECT OF FEEDING DIFFERENT LEVELS OF DIETARY INORGANIC SULFATE UPON THE ACTIVITY OF RAT LIVER PROPIONYL CoA CARBOXYLASE[1]

Percent dietary sulfate	Propionyl CoA carboxylase activity	
	n moles/mg N[2]	
0.0002	362 ± 21[a3]	(5)[4]
0.02	230 ± 45	(5)
0.10	388 ± 55[a]	(5)
0.42	454 ± 46[a]	(5)

[1]The reaction mixture contained 0.1 ml of a 10% liver homogenate, 60 μ moles Tris-HCl, pH 8.5, 9.4 μ moles $NaH^{14}CO_3$ diluted to give a specific activity of 3.4×10^4 cpm/μ mole bicarbonate, 2.0 μ moles Na_2ATP, 2.5 μ moles reduced glutathione, 5.0 μ moles $MgCl_2$, 75 μ moles KCl, 1.0 μ moles propionyl CoA in a final volume of 0.75 ml.

[2]Values are means ± SEM.

[3]All means not followed by the same superscript are significantly different ($P<0.05$) by the method of Duncan's new multiple range test for equal replications (50).

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

TABLE 7

EFFECT OF FEEDING DIFFERENT LEVELS OF DIETARY INORGANIC SULFATE ON THE ACTIVITY OF RAT LIVER PROPIONYL CoA CARBOXYLASE WHEN SODIUM PROPIONATE WAS SUBSTITUTED FOR PROPIONYL CoA IN THE REACTION MIXTURE[1]

Percent dietary sulfate	Propionyl CoA carboxylase activity	
	n moles/mg N[2]	
0.0002	91 ± 23[b3]	(5)[4]
0.02	8 ± 6[a]	(5)
0.10	74 ± 19[b]	(5)
0.42	43 ± 14[ab]	(5)

[1]The reaction mixture is the same as that in Table 6 except that 3.1 μ moles of sodium propionate were substituted for 1.0 μ mole propionyl CoA.

[2]Values are means ± SEM.

[3]All means not followed by the same superscript are significantly different ($P<0.05$) by the method of Duncan's new multiple range test for equal replications (50).

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

TABLE 8

EFFECT OF FEEDING DIFFERENT LEVELS OF DIETARY INORGANIC SULFATE UPON THE ACTIVITY OF RAT LIVER PROPIONYL CoA CARBOXYLASE WHEN SODIUM PROPIONATE PLUS COENZYME A WERE SUBSTITUTED FOR PROPIONYL CoA IN THE REACTION MIXTURE

Percent dietary sulfate	Propionyl CoA carboxylase activity
	n moles/mg N[2]
0.0002	99 ± 21[a][3] (5)[4]
0.02	14 ± 12 (5)
0.10	110 ± 18[a] (5)
0.42	121 ± 27[a] (5)

[1]The reaction mixture is the same as that in Table 6 except that 3.1 μ moles of sodium propionate plus 1.9 μ moles of coenzyme A were substituted for 1.0 μ mole of propionyl CoA.

[2]Values are means ± SEM.

[3]All means not followed by the same superscript are significantly different ($P<0.01$) by the methods of Duncan's new multiple range test for equal replications (50).

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

78% in the rate of the reaction was observed. However, once again the pattern of increased enzyme activity, observed when inorganic sulfate was fed at levels above or below the optimal dietary level, remained unchanged.

II. MEASUREMENT OF REDUCED GLUTATHIONE EFFECT

Reduced glutathione was omitted from the reaction mixture in an attempt to determine whether or not dietary inorganic sulfate increased propionyl CoA carboxylase activity through an effect upon tissue levels of reduced glutathione. A comparison of the data presented in Tables 9 and 6 was made. A decrease in the rate of the reaction was again evident although the pattern of increased enzymatic activity, observed when inorganic sulfate was fed at levels above or below the established dietary optimum, remained.

The data presented in Tables 6 through 9 show that the pattern of increased activation of propionyl CoA carboxylase, observed at levels of dietary inorganic sulfate supplementation above or below the established dietary optimum of 0.02%, could not be explained by the effect of dietary inorganic sulfate upon tissue levels of CoA, the acylation of propionate or tissue levels of reduced glutathione. Alternative explanations for the mechanism of this sulfate effect were sought.

TABLE 9

EFFECT OF FEEDING DIFFERENT LEVELS OF DIETARY INORGANIC SULFATE UPON THE ACTIVITY OF RAT LIVER PROPIONYL CoA CARBOXYLASE WHEN REDUCED GLUTATHIONE WAS OMITTED FROM THE REACTION MIXTURE[1]

Percent dietary sulfate	Propionyl CoA carboxylase activity	
	n moles/mg N[2]	
0.0002	222 ± 17[ab3]	(2)[4]
0.02	57 ± 55[a]	(2)
0.10	351 ± 80[b]	(2)
0.42	328 ± 18[b]	(2)

[1]The reaction mixture is the same as that in Table 6 except that reduced glutathione was omitted from the reaction mixture.

[2]Values are means ± SEM.

[3]All means not followed by the same superscript are significantly different ($P<0.05$) by the method of Duncan's new multiple range test for equal replications (50).

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

III. MEASUREMENT OF THE EFFECT OF DIETARY INORGANIC SULFATE UPON A CYTOPLASMIC FACTOR NECESSARY FOR ENZYME SYNTHESIS

In 1974, Murthy and Mistry[6] reported the isolation of a cytoplasmic factor necessary for the synthesis of propionyl CoA carboxylase. The possibility that the level of inorganic sulfate in the diet affected the availability of this cytoplasmic factor was investigated.

The data presented in Table 10 are the results of a preliminary experiment designed to reveal the level of dietary inorganic sulfate supplementation at which the availability of a cytoplasmic factor necessary for enzyme synthesis might be affected. The mitochondrial-cytoplasmic combination from rats fed 0.02% of dietary inorganic sulfate (combination C) served as the control. There was a 25% increase in enzyme activity when the cytoplasm from rats fed 0.0002% of dietary inorganic sulfate was combined with the mitochondria from rats fed 0.02% of dietary inorganic sulfate (combination B). The combination of the cytoplasm from rats fed 0.42% of dietary inorganic sulfate with the mitochondria from rats fed 0.0002% (combination A), 0.02% (combination D), or 0.42% (combination H) of dietary inorganic sulfate caused little change in enzyme activity. Further experimentation was restricted, therefore, to mitochondrial-cytoplasmic combinations from rats fed 0.0002% and 0.02% of dietary inorganic sulfate.

Liver mitochondria and liver cytoplasm from rats fed 0.0002% and 0.02% of dietary inorganic sulfate were combined as shown in Table 11. The cytoplasm from rats fed 0.0002% of dietary inorganic sulfate when combined with the mitochondria from rats fed 0.02% of dietary

TABLE 10

SURVEY OF THE EFFECT OF FEEDING DIFFERENT LEVELS OF DIETARY INORGANIC SULFATE UPON THE AVAILABILITY OF A CYTOPLASMIC FACTOR NECESSARY FOR PROPIONYL CoA CARBOXYLASE SYNTHESIS[1]

Designator	Liver mitochondria	Liver cytoplasm	Propionyl CoA carboxylase activity
	rats fed % of dietary $SO_4^=$	rats fed % of dietary $SO_4^=$	n moles/mg N[2]
A	0.0002	0.42	46
B	0.02	0.0002	55
C	0.02	0.02	44
D	0.02	0.42	48
E	0.10	0.02	35
F	0.10	0.42	30
G	0.42	0.02	47
H	0.42	0.42	41

[1]The reaction mixture contained 0.1 ml of the mitochondrial-cytoplasmic combinations shown above, 60 µ moles Tris-HCl, pH 7.5, 9.4 µ moles $NaH^{14}CO_3$ diluted to give a specific activity of 9.6×10^4 cpm/µ mole bicarbonate, 2.0 µ moles Na_2ATP, 2.5 µ moles reduced glutathione, 5.0 µ moles $MgCl_2$, 75 µ moles KCl, 5 µ moles $Na_3C_6H_5O_7 \cdot 2H_2O$, 1 µ mole propionyl CoA in a final volume of 0.75 ml.

[2]Values are averages of duplicate determinations in a model experiment.

TABLE 11

EFFECT OF FEEDING DIFFERENT LEVELS OF DIETARY INORGANIC SULFATE UPON THE AVAILABILITY OF A CYTOPLASMIC FACTOR NECESSARY FOR PROPIONYL CoA CARBOXYLASE SYNTHESIS[1]

Designator	Liver mitochondria	Liver cytoplasm	Propionyl CoA carboxylase activity
	rats fed % of dietary $SO_4^=$	rats fed % of dietary $SO_4^=$	n moles/mg N[2]
Q	0.0002	0.0002	13.8 ± 1.0^{b}[3] (5)[4]
R	0.0002	0.02	11.2 ± 0.9^{ac} (5)
S	0.02	0.0002	12.9 ± 0.3^{bc} (5)
T	0.02	0.02	9.2 ± 0.1^{a} (5)

[1]The reaction mixture contained 0.1 ml of the mitochondrial-cytoplasmic combinations shown above, plus the remaining constituents as shown in Table 10.

[2]Values are means ± SEM.

[3]All means not followed by the same superscript are significantly different ($P<0.05$) by the method of Duncan's new multiple range test for equal replications (50).

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

inorganic sulfate (combination S) caused a significant increase ($P<0.05$) in the activity of propionyl CoA carboxylase when compared to the mitochondrial-cytoplasmic combination from rats fed 0.02% of dietary inorganic sulfate (combination T). The cytoplasm from rats fed 0.02% of dietary inorganic sulfate when combined with the mitochondria from rats fed 0.0002% of dietary inorganic sulfate (combination R) caused a significant decrease ($P<0.05$) in enzyme activity when compared with the mitochondrial-cytoplasmic combinations from rats fed 0.0002% of dietary inorganic sulfate (combination Q). Propionyl CoA carboxylase activity was significantly greater ($P<0.05$) in the mitochondrial-cytoplasmic combinations from rats fed 0.0002% of dietary inorganic sulfate (combination Q) than in the mitochondrial-cytoplasmic combinations from rats fed 0.02% of dietary inorganic sulfate (combination T).

The addition of biotin to the reaction mixture caused a complete reversal of this phenomenon (Table 12). When biotin was added to the reaction mixture, the cytoplasm from rats fed 0.02% of dietary inorganic sulfate combined with the mitochondria from rats fed 0.0002% of dietary inorganic sulfate (combination X) caused a significant increase ($P<0.05$) in propionyl CoA carboxylase activity when compared to the mitochondrial-cytoplasmic combination from rats fed 0.0002% of dietary inorganic sulfate (combination W). The addition of the cytoplasm from rats fed 0.0002% of dietary inorganic sulfate to the mitochondria from rats fed 0.02% of dietary inorganic sulfate (combination Y) caused a significant decrease ($P<0.05$) in enzyme activity when compared to the mitochondrial-cytoplasmic combinations from rats fed 0.02% of dietary

TABLE 12

EFFECT OF FEEDING DIFFERENT LEVELS OF DIETARY INORGANIC SULFATE UPON THE AVAILABILITY OF A CYTOPLASMIC FACTOR NECESSARY FOR PROPIONYL CoA CARBOXYLASE SYNTHESIS WHEN BIOTIN WAS ADDED TO THE REACTION MIXTURE[1]

Designator	Liver mitochondria	Liver cytoplasm	Propionyl CoA carboxylase activity
	rats fed % of dietary $SO_4^=$	rats fed % of dietary $SO_4^=$	n moles/mg N[2]
W	0.0002	0.0002	24.2 ± 0.9^{b}[3] (4)[4]
X	0.0002	0.02	31.1 ± 2.8^{ac} (4)
Y	0.02	0.0002	24.8 ± 1.7^{bc} (4)
Z	0.02	0.02	34.0 ± 2.6^{a} (4)

[1]The reaction mixture is the same as that in Table 11 except that 0.5 μ mole of biotin was added.

[2]Values are means ± SEM.

[3]All means not followed by the same superscript are significantly different ($P<0.05$) by the method of Duncan's new multiple range test for equal replications (50).

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

inorganic sulfate (combination Z). Propionyl CoA carboxylase activity was significantly greater ($P<0.05$) in the mitochondrial-cytoplasmic combinations from rats fed 0.02% of dietary inorganic sulfate (combination Z) than in the mitochondrial-cytoplasmic combinations from rats fed 0.0002% of dietary inorganic sulfate (combination W).

IV. MEASUREMENT OF THE EFFECT OF INORGANIC SULFATE UPON THE ENZYME PROTEIN (KINETICS OF THE PROPIONYL CoA CARBOXYLASE REACTION)

Different levels of sodium sulfate were added to the reaction mixture in an attempt to elucidate the mechanism of the sulfate effect when inorganic sulfate was fed at levels above the optimal. The addition of from 1.87 to 30.00 μ moles of sodium sulfate to the reaction mixture caused a gradual and eventually significant increase ($P<0.05$) in the activity of rat liver propionyl CoA carboxylase (Table 13).

The inclusion of biotin in the reaction mixture, when graded amounts of sodium sulfate were added, also produced a gradual and eventually significant ($P<0.05$) increase in enzyme activity (Table 14.) However, when biotin was included in the reaction mixture, the amount of sulfate required to produce a significant increase in enzyme activity (5.62 μ moles) was 63% less than that required (15.00 μ moles) when no biotin was added.

The possibility that inorganic sulfate is an allosteric activator of propionyl CoA carboxylase was investigated since the enzyme is composed of 4 equivalent subunits (31-34) that have been shown to

TABLE 13

EFFECT OF ADDING DIFFERENT LEVELS OF INORGANIC SULFATE TO THE REACTION MIXTURE UPON THE ACTIVITY OF RAT LIVER PROPIONYL CoA CARBOXYLASE[1]

Concentration of $SO_4^=$	Propionyl CoA carboxylase activity
μ moles/tube	n moles/mg N[2]
0.00	297 ± 12[a][3] (5)[4]
1.87	295 ± 9[a] (5)
3.75	301 ± 12[a] (5)
5.62	305 ± 11[ab] (5)
7.50	322 ± 3[abc] (5)
15.00	329 ± 4[bc] (5)
22.50	336 ± 5[bc] (5)
30.00	337 ± 0[bc] (5)

[1]The reaction mixture is the same as that in Table 6 except that 9.4 μ moles $NaH^{14}CO_3$ were diluted to give a specific activity of 9.6×10^4 cpm/μ mole bicarbonate.

[2]Values are means ± SEM.

[3]All means not followed by the same superscript are significantly different ($P<0.05$) by the method of Duncan's new multiple range test for equal replications (50).

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

TABLE 14

EFFECT OF ADDING DIFFERENT LEVELS OF INORGANIC SULFATE TO THE REACTION MIXTURE UPON THE ACTIVITY OF RAT LIVER PROPIONYL CoA CARBOXYLASE WHEN BIOTIN WAS INCLUDED IN THE REACTION MIXTURE[1]

Concentration of $SO_4^=$	Propionyl CoA carboxylase activity	
μ moles/tube	n moles/mg N[2]	
0.00	311 ± 0[a][3]	(4)[4]
1.87	326 ± 3[ab]	(4)
3.75	327 ± 2[ab]	(4)
5.62	340 ± 13[b]	(4)
7.50	341 ± 11[b]	(4)

[1]The reaction mixture is the same as that in Table 13 except that 0.5 μ mole of biotin was added.

[2]Values are means ± SEM.

[3]All means not followed by the same superscript are significantly different ($P<0.05$) by the method of Duncan's new multiple range test for equal replications (50).

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

undergo conformational changes associated with the binding of substrates and effectors (37). Propionyl CoA carboxylase, therefore, meets several of the basic criteria of an allosteric enzyme (51).

A kinetic analysis of the enzyme protein was performed. The sigmoid character of the substrate-saturation curve of propionyl CoA carboxylase in the absence of inorganic sulfate and the sigmoid response of propionyl CoA carboxylase in the presence of inorganic sulfate (Figure 5) strongly suggest that the mechanism of the sulfate effect observed when inorganic sulfate is fed at levels above the established dietary optimum (0.02%) is one of allosteric activation.

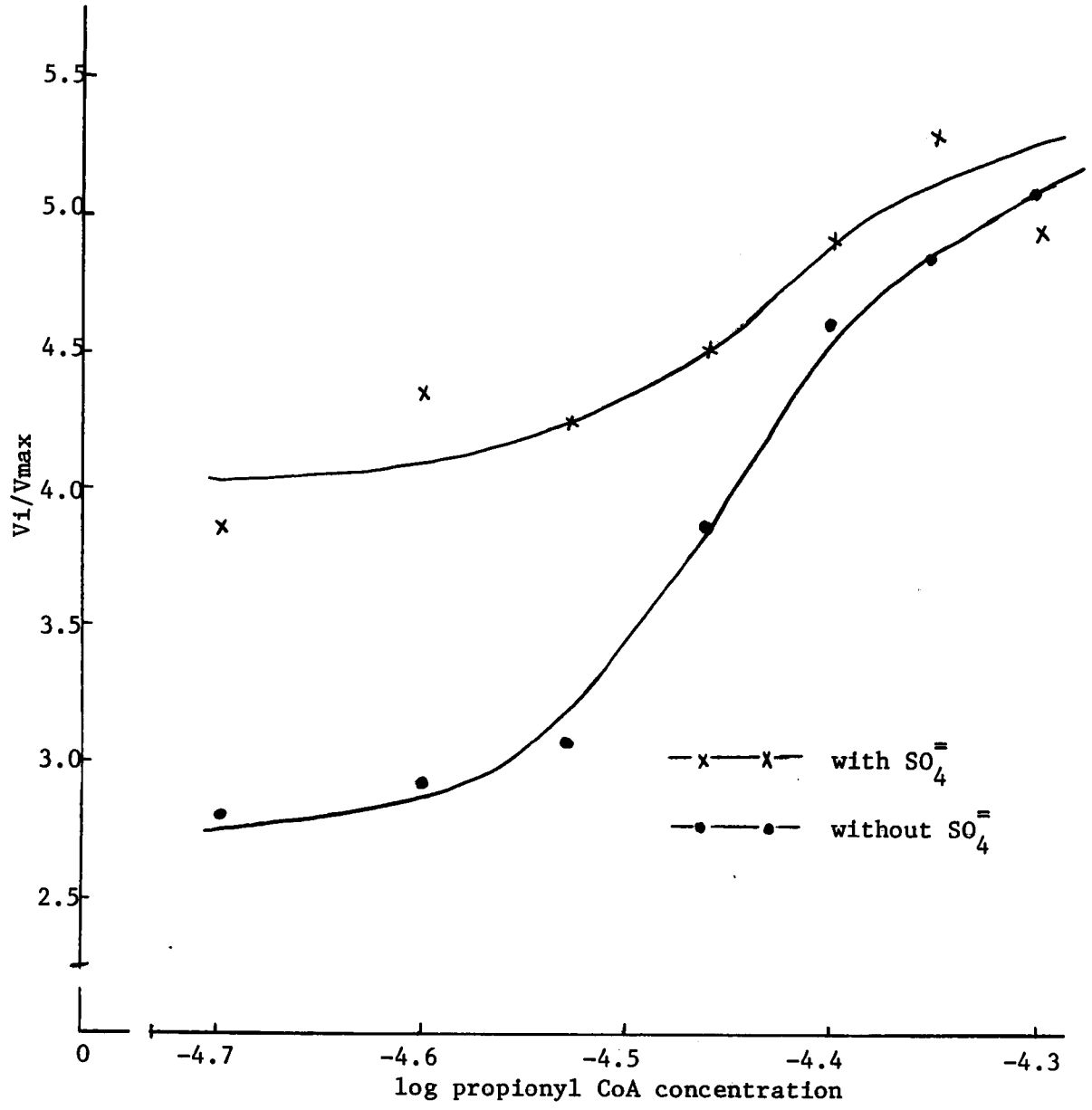

Figure 5. Substrate-saturation curves of propionyl CoA carboxylase in the presence and absence of sulfate.

CHAPTER V

DISCUSSION

Data have been presented (Table 6, page 33) which show that inorganic sulfate, when fed at levels above or below the established dietary optimum of 0.02%, caused a significant increase in the activity of propionyl CoA carboxylase. Attempts were made, therefore, to elucidate the mechanism by which inorganic sulfate exerted this effect.

Verma[4] demonstrated that very low levels of dietary inorganic sulfate caused a decrease in liver levels of coenzyme A. It was postulated, therefore, that the mechanism by which inorganic sulfate exerted an effect upon propionyl CoA carboxylase activity was related to the effect of the level of dietary inorganic sulfate upon tissue levels of CoA and thus upon the acylation of propionate. The data presented in Tables 7 and 8, pages 34 and 36, however, did not support this theory. Although the rate of the propionyl CoA carboxylase reaction was decreased when sodium propionate or sodium propionate plus CoA was substituted for propionyl CoA in the reaction mixture, the basic pattern of increased enzymatic activity observed at levels of dietary inorganic sulfate supplementation above or below the optimum was unchanged. These findings indicated, therefore, that the effect of inorganic sulfate upon liver levels of CoA and/or propionate acylation was not the mechanism by which inorganic sulfate increased the activity of propionyl CoA carboxylase.

Since propionyl CoA carboxylase is an enzyme which requires the integrity of sulfhydryl groups to function (28, 32, 38), an attempt was made to relate the observed increase in enzymatic activity when inorganic sulfate was fed at levels above or below the established dietary optimum to an effect of the level of dietary inorganic sulfate upon tissue levels of reduced glutathione. However, when reduced glutathione was omitted from the reaction mixture (Table 9, page 37), the observed pattern of increased enzymatic activity at levels of dietary inorganic sulfate supplementation above or below the optimum was unaffected. Thus, the observed activation of propionyl CoA carboxylase was not explained by an effect of inorganic sulfate upon tissue levels of reduced glutathione.

The data presented in Tables 7, 8, and 9, pages 34, 36, and 37, ruled out the possibility that the mechanism of the effect of the level of dietary inorganic sulfate upon the activity of propionyl CoA carboxylase was an effect of inorganic sulfate upon tissue levels of CoA, the acylation of propionate or tissue levels of reduced glutathione. Alternate explanations for the mechanism by which the level of dietary inorganic sulfate increased the activity of propionyl CoA carboxylase were investigated.

In addition to holocarboxylase synthetase and the apocarboxylase, Murthy and Mistry[6] isolated a cytoplasmic factor required for the synthesis of propionyl CoA carboxylase. The effect of the level of dietary inorganic sulfate upon the availability of this cytoplasmic factor was investigated. Data presented in Table 11, page 40, show that the cytoplasm from rats fed 0.0002% of dietary inorganic sulfate when

combined with the mitochondria from rats fed 0.02% of dietary inorganic sulfate caused a significant increase in the activity of propionyl CoA carboxylase when compared to mitochondrial-cytoplasmic combinations from rats fed 0.02% of dietary inorganic sulfate, while the cytoplasm from rats fed 0.02% of dietary inorganic sulfate when combined with the mitochondria from rats fed 0.0002% of dietary inorganic sulfate caused a significant decrease in the activity of propionyl CoA carboxylase when compared to mitochondrial-cytoplasmic combinations from rats fed 0.0002% of dietary inorganic sulfate. These data suggested, therefore, that the mechanism of the effect of very low levels of dietary inorganic sulfate (0.0002%) upon enzyme activity was related to an increased availability of a cytoplasmic factor necessary for enzyme synthesis.

The addition of biotin to the mitochondrial-cytoplasmic combinations mentioned above (Table 12, page 42) completely reversed the pattern of enzymatic activation observed previously (Table 11, page 40). The cytoplasm from rats fed 0.02% of dietary inorganic sulfate when combined with the mitochondria from rats fed 0.0002% of dietary inorganic sulfate now caused a significant increase in enzymatic activity when compared to mitochondrial-cytoplasmic combinations from rats fed 0.0002% of dietary inorganic sulfate, while the cytoplasm from rats fed 0.0002% of dietary inorganic sulfate when combined with the mitochondria from rats fed 0.02% of dietary inorganic sulfate caused a significant decrease in enzyme activity when compared with mitochondrial-cytoplasmic combinations from rats fed 0.02% of dietary inorganic sulfate. Thus, a very low level of dietary inorganic sulfate (0.0002%)

apparently increased the availability of biotin for enzyme synthesis possibly by increasing the formation of biotinyl-AMP or by increasing the rate of binding of biotin to the epsilon amino group of the lysine residues of the enzyme protein.

The mechanism of the increase in enzymatic activity observed when inorganic sulfate was fed at levels above the dietary optimum was also investigated. The data presented in Table 13, page 44, revealed that the addition of graded amounts of inorganic sulfate to the reaction mixture caused a gradual and eventually significant increase in enzyme activity. Horne and Nordlie (52) observed a similar increase in the activity of glucose dehydrogenase when from 30 to 90 μ moles of inorganic sulfate were added to the reaction mixture. The addition of biotin to the reaction mixture (Table 14, page 45) caused a 63% reduction in the amount of inorganic sulfate required to produce a significant increase in enzyme activity. Madappally and Mistry (53) showed that the addition of from 150 to 300 μ moles of inorganic sulfate to the reaction mixture protected chicken liver pyruvate carboxylase from avidin inhibition.

A kinetic study of the enzyme protein (Figure 5, page 47) indicated that inorganic sulfate allosterically activated propionyl CoA carboxylase. This activation could have been accomplished by a change in the configuration of the enzyme protein in such a way that the biotin binding sites became more accessible and thus the rate of biotin binding increased; in other words, inorganic sulfate could cause the expression of an increased number of biotin binding sites on the enzyme protein itself.

These data indicate, therefore, that inorganic sulfate plays a role in biotin metabolism.

It seems unusual that both very low (0.0002%) and high levels (0.10% and 0.42%) of dietary inorganic sulfate would produce the same effect, an increase in the activity of propionyl CoA carboxylase. However, an increase in enzyme activity when inorganic sulfate is fed at very low levels (0.0002%) is consistent with the findings of Michels and Smith (1) that very low levels of dietary inorganic sulfate appear to increase the methionine requirement. The carbon skeleton of methionine is metabolized via conversion to propionate (15); thus, conditions which cause an increased utilization of methionine could also cause an increased activation of the enzymes responsible for its catabolism.

Increases in blood glucose levels (3), in liver glycogen levels[2] and in the activity of several key enzymes of carbohydrate metabolism[3] were observed when inorganic sulfate comprised 0.10% or 0.42% of the diet. Caster and Bleeker (54) showed that feeding small amounts of certain branched chain amino acids, which are metabolized to propionate in mammals, produced hyperglycemia in rats. The approximate inorganic sulfate content of the diets fed by Caster and Bleeker was 0.10%, a level which in the present investigation resulted in a significant activation of propionyl CoA carboxylase. If levels of dietary inorganic sulfate supplementation above the optimum increase the rate of gluconeogenesis, as is suggested by the above reports, then the increased activation of propionyl CoA carboxylase under these circumstances is reasonable since the carbon skeletons of valine (13, 14),

isoleucine (14), threonine and methionine (15) are catabolized via conversion to propionate.

The data presented in this investigation, therefore, serve to strengthen the concept of inorganic sulfate as a metabolic regulator and show that in this instance, the mechanism of the inorganic sulfate effect is related to an effect of inorganic sulfate upon biotin metabolism.

CHAPTER VI

SUMMARY

The relationship between the level of dietary inorganic sulfate and the activity of rat liver propionyl CoA carboxylase was investigated. An increase in the activity of propionyl CoA carboxylase was observed when inorganic sulfate was fed at levels above or below the established dietary optimum of 0.02%. However, the mechanism by which inorganic sulfate exerted this effect was different in each case. When fed at levels below the established dietary optimum (0.0002%), inorganic sulfate increased the availability of a cytoplasmic factor necessary for enzyme synthesis. Kinetic studies of the enzyme protein indicated that high levels of dietary inorganic sulfate (0.10% and 0.42%) caused an allosteric activation of the enzyme. In both cases, the effect of the level of dietary inorganic sulfate upon enzyme activity was related to an effect of inorganic sulfate upon biotin metabolism.

Increases in propionyl CoA carboxylase activity at levels of dietary inorganic sulfate supplementation above and below the optimum were explained by the fact that very low levels of dietary inorganic sulfate (0.0002%) appear to increase the requirement for methionine while high levels of dietary inorganic sulfate (0.10% and 0.42%) appear to increase the rate of gluconeogenesis. In both cases, a breakdown of amino acids is involved and the catabolism of the carbon skeletons of valine, isoleucine, methionine, and threonine via their conversion to propionate is well documented.

The data presented in this investigation, therefore, serve to strengthen the concept of inorganic sulfate as a metabolic regulator and show that the mechanism of the sulfate effect observed in this investigation, an activation of propionyl CoA carboxylase, is related to an effect of inorganic sulfate upon biotin metabolism.

LITERATURE CITED

LITERATURE CITED

1. Michels, F. G. & Smith, J. T. (1965) A comparison of the utilization of organic and inorganic sulfur by the rat. J. Nutr. *87*, 217-220.

2. Whittle, B. & Smith, J. T. (1974) Effect of dietary sulfur on taurine excretion by the rat. J. Nutr. *104*, 666-670.

3. Feland, B., Fuqua, E. G., & Smith, J. T. (1973) Effect of dietary sulfur on serum cholesterol and the glycocholic:taurocholic acid ratio of the rat. J. Nutr. *103*, 1561-1565.

4. Brown, R. G., Button, G. M., & Smith, J. T. (1965) The effect of sulfate deficiency on the mechanical strength of the rat's aorta. Biochim. et Biophys. Acta *101*, 361-363.

5. Wellers, G., Boille, G., & Chevan, J. (1960) Metabolism of sulfur. IV. Partial replacement of the endogenous loss by sodium sulfate in the adult white rat. J. Physiol. (Paris) *52*, 903-910.

6. Button, G. M., Brown, R. G., Michels, F. G., & Smith, J. T. (1965) Utilization of calcium and sodium sulfate by the rat. J. Nutr. *87*, 211-216.

7. Roe, D. A. (1971) Effects of methionine and inorganic sulfate on indole toxicity and indican excretion in rats. J. Nutr. *101*, 645-653.

8. Smith, J. T. (1973) An optimal level of inorganic sulfate for the diet of a rat. J. Nutr. *103*, 1008-1011.

9. Alberts, A. W. & Vagelos, P. R. (1972) Propionyl-CoA carboxylase. In: The Enzymes (Boyer, P. D., ed.) pp. 46-53, Academic Press, New York.

10. Deuel, H. J., Butts, J. S., Hallman, L. F., & Cutler, C. H. (1935) Studies on ketosis. VII. Quantitative studies on β-oxidation. Glycogen formation from various fatty acids. J. Biol. Chem. *112*, 15-23.

11. Atchley, W. A. (1948) Studies on the cyclophorase system. IV. Direct demonstration of β-oxidation. J. Biol. Chem. *176*, 123-131.

12. Lardy, H. A. (1952) A theory concerning the mechanism of fatty acid oxidation and synthesis, and of carbon dioxide fixation. Proc. Nat. Acad. Sci. U.S.A. *38*, 1003-1013.

13. Kinnory, D. S., Takeda, Y., & Greenberg, D. M. (1955) Isotope studies on the metabolism of valine. J. Biol. Chem. *212*, 385-396.

14. Coon, M. J., Robinson, W. G., & Backhawat, B. K. (1955) Enzymatic studies on the biological degradation of the branched chain amino acids. In: Amino Acid Metabolism (McElroy, W. D. & Glass, H. B., eds.) pp. 431-441, John Hopkins Press, Baltimore.

15. Cantarow, A. & Shepartz, B. (1967) XX. Metabolism of nucleic acids and related substances. XXI. Metabolism of proteins. In: Biochemistry, 4th ed. (Cantarow, A. & Shepartz, B., eds.) pp. 523 & 576, W. B. Saunders Company, Philadelphia.

16. Hutton, D. & Steinberg, D. (1973) Identification of propionate as a degradation product of phytanic acid oxidation in rat and human tissues. J. Biol. Chem. *248*, 6871-6875.

17. Ringer, A. I. (1912) The chemistry of gluconeogenesis. I. The quantitative conversion of propionic acid into glucose. J. Biol. Chem. *12*, 511-515.

18. Huennekens, F. M., Mahler, H. R., & Nordmann, J. (1951) Studies on the cyclophorase system. XVI. The oxidation of propionic acid. Arch. Biochem. Biophys. *30*, 66-76.

19. Mahler, H. R. & Huennekens, F. M. (1953) The pathway of propionate oxidation. Biochim. et Biophys. Acta *11*, 575-583.

20. Lorber, V., Lifson, N., Sakami, W., & Wood, H. G. (1950) Conversion of propionate to liver glycogen in the intact rat, studied with isotopic propionate. J. Biol. Chem. *183*, 531-538.

21. Daus, L., Meinke, M., & Calvin, M. (1952) Propionic acid metabolism in mouse liver slices. J. Biol. Chem. *196*, 77-87.

22. Shreeve, W. (1952) Conversion of C^{14}-labeled propionate, lactate and pyruvate to acetyl groups in the rat. J. Biol. Chem. *195*, 1-10.

23. Lardy, H. A. & Peanasky, R. (1953) Metabolic functions of biotin. Physiol. Rev. *33*, 560-565.

24. Lardy, H. A. & Adler, J. (1956) Synthesis of succinate from propionate and bicarbonate by soluble enzymes from liver mitochondria. J. Biol. Chem. *219*, 933-942.

25. Flavin, M. & Ochoa, S. (1956) Metabolism of propionic acid in animal tissues. I. Enzymic conversion of propionate to succinate. J. Biol. Chem. *229*, 965-979.

26. Katz, J. & Chaikoff, I. L. (1955) The metabolism of propionate by rat liver slices and the formation of isosuccinic acid. J. Am. Chem. Soc. *77*, 2659-2660.

27. Flavin, M., Ortiz, P. J., & Ochoa, S. (1955) Metabolism of propionic acid in animal tissues. Nature *176*, 823-826.

28. Tietz, A. & Ochoa, S. (1959) Metabolism of propionic acid in animal tissues. V. Purification and properties of propionyl carboxylase. J. Biol. Chem. *234*, 1394-1400.

29. Friedberg, F., Adler, J., & Lardy, H. A. (1956) The carboxylation of propionic acid by liver mitochondria. J. Biol. Chem. *219*, 943-950.

30. Scholte, H. R. (1969) Intracellular and intramitochondrial distribution of malonyl CoA decarboxylase and propionyl CoA carboxylase in rat liver. Biochim. et Biophys. Acta *178*, 137-144.

31. Kaziro, Y. (1969) Crystalline propionyl-CoA carboxylase from pig heart. In: Methods in Enzymology. XIII. Citric Acid Cycle (Lowenstein, J. M., ed.) pp. 181-190, Academic Press, New York.

32. Kaziro, Y., Ochoa, S., Warner, R. C., & Chen, J-Y. (1961) Metabolism of propionic acid in animal tissues. VIII. Crystalline propionyl carboxylase. J. Biol. Chem. *236*, 1917-1923.

33. Kaziro, Y., Grossman, A., & Ochoa, S. (1965) Metabolism of propionic acid in animal tissues. XI. Further studies on crystalline propionyl coenzyme A carboxylase. J. Biol. Chem. *240*, 64-67.

34. Moss, J. & Lane, M. D. (1971) The biotin-dependent enzymes. In: Advances in Enzymology (Nord, F. F., ed.) pp. 378-384, Interscience Publishers, New York.

35. Kaziro, Y., Leone, E., & Ochoa, S. (1960) Biotin and propionyl carboxylase. Proc. Nat. Acad. Sci. U.S.A. *46*, 1319-1327.

36. Neujahr, H. Y. (1963) The influence of univalent cations on crystalline propionyl CoA carboxylase. Acta Chem. Scand. *17*, 1140-1150.

37. Edwards, J. B. & Keech, D. B. (1968) Activation of pig heart propionyl-CoA carboxylase by potassium ions. Biochim. et Biophys. Acta *159*, 167-175.

38. Edwards, J. B. & Keech D. B. (1967) The essential thiol group of propionyl-CoA carboxylase. Biochim. et Biophys. Acta *146*, 576-583.

39. Kaziro, Y. & Ochoa, S. (1961) Mechanism of the propionyl carboxylase reaction. I. Carboxylation and decarboxylation of the enzyme. J. Biol. Chem. *236*, 3131-3136.

40. Yoshito, K., Hass, L. F., Boyer, P. D., & Ochoa, S. (1962) Mechanism of the propionyl carboxylase reaction. II. Isotopic exchange and tracer experiments. J. Biol. Chem. *237*, 1460-1468.

41. Ochoa, S. & Kaziro, Y. (1961) Biotin enzymes. Fed. Proc. *20*, 982-988.

42. Kosow, D. P. & Lane, M. D. (1961) Propionyl apocarboxylase activation catalyzed by cell free enzyme extracts. Biochem. Biophys. Res. Commun. *5*, 191-195.

43. Kosow, D. P., Huang, S. C., & Lane, M. D. (1962) Propionyl holocarboxylase synthesis. I. Preparation and properties of the enzyme system. J. Biol. Chem. *237*, 3633-3637.

44. Siegel, L., Foote, J. L., Christner, J. E., & Coon, M. J. (1963) Propionyl CoA holocarboxylase synthesis from biotinyl adenylate and the apocarboxylase in the presence of an activating enzyme. Biochem. Biophys. Res. Commun. *13*, 307-312.

45. Siegel, L., Foote, J. L., & Coon, M. J. (1965) The enzymic synthesis of propionyl coenzyme A holocarboxylase from d-biotinyl -5′- adenylate and the apocarboxylase. J. Biol. Chem. *240*, 1025-1031.

46. Georgio, A. J. & Whitaker, T. R. (1973) Some properties of propionyl CoA carboxylase partially purified from human liver. Biochem. Med. *7*, 473-478.

47. Caputto, R., McCay, R. B., & Carpenter, M. P. (1958) Requirements for Mn^{++} and Co^{++} for the synthesis of ascorbic acid by liver extracts of animals deprived of tocopherol. J. Biol. Chem. *233*, 1025-1029.

48. Hubbell, R. B., Mendel, L. B., & Wakeman, A. J. (1937) A new salt mixture for use in experimental diets. J. Nutr. *14*, 273-285.

49. Willets, C. O. & Ogg, C. L. (1950) Report on standardization of microchemical methods. Micro-Kjeldahl nitrogen determination. J. Assoc. Official Agri. Chem. *33*, 179-188.

50. Steel, R. G. D. & Torrie, J. H. (1960) Principles and Procedures of Statistics, McGrow-Hill Book Company, Inc., New York.

51. Kirschner, K. (1968) Allosteric regulation of enzyme activity. An introduction to the molecular basis of and the experimental approaches to the problem. Current Top. Microbiol. Immunol. *44*, 123-146.

52. Horne, R. N. & Nordlie, R. C. (1971) Activation by bicarbonate, orthophosphate and sulfate of rat liver microsomal glucose dehydrogenase. Biochim. et Biophys. Acta *242*, 1-13.

53. Madappally, M. M. & Mistry, S. P. (1970) Protection of chicken liver pyruvate carboxylase by sulphate ions against avidin inhibition. Life Sci. *9*, 833-839.

54. Caster, W. O. & Bleeker, S. (1975) Hyperglycemic effect of amino compounds structurally related to caproate in rats. J. Nutr. *105*, 308-310.

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