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Effect of Sulfur Nutrition on the Activity of Acetyl Coenzyme A Carboxylase, Citrate Cleavage Enzyme, and Malic Enzyme in Rat Liver

Joyce Brann Bittle
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Joyce Brann Bittle entitled "Effect of Sulfur Nutrition on the Activity of Acetyl Coenzyme A Carboxylase, Citrate Cleavage Enzyme, and Malic Enzyme in Rat Liver." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

John T. Smith, Major Professor

We have read this dissertation and recommend its acceptance:

Jane R. Savage, Irshad Ahmad, Mary Nelle Traylor

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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and recommend its acceptance:

Jane R. Savage

Farhad Ahmed

Mary N. Traylor

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

EFFECT OF SULFUR NUTRITION ON THE ACTIVITY OF ACETYL COENZYME A CARBOXYLASE, CITRATE CLEAVAGE ENZYME, AND MALIC ENZYME IN RAT LIVER

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee

Joyce Brann Bittle

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ABSTRACT

The level of dietary inorganic sulfate as well as the nature of the neutral sulfate fed a rat have been shown to affect the activity of certain key enzymes of carbohydrate metabolism. Metabolic alterations have been demonstrated in rats fed diets containing inorganic sulfate at levels above or below the established optimal level of 0.02%. The present investigation was designed to extend these studies to include lipogenesis.

Ninety-two adult male rats were fed diets containing 15% casein and supplemented with $CaSO_4$, methionine, and cysteine in order to prepare diets with different inorganic sulfate levels and different neutral to inorganic sulfate ratios. These diets were fed for a period of 17 days. At the end of the dietary period the rats were sacrificed by decapitation and their livers removed for enzyme analyses. Acetyl CoA carboxylase activity was measured by a fixation of $^{14}CO_2$ from $H^{14}CO_3^-$, citrate cleavage enzyme activity was measured by coupling the reaction catalyzed by citrate cleavage enzyme with the reduction of oxalacetate with malic dehydrogenase and then following the change of absorbance of NADH to NAD, and malic enzyme was measured by following the change of absorbance of NADP to NADPH.

When diets containing constant levels of total sulfur as sulfate supplemented with inverse amounts of methionine and $CaSO_4$ were fed, a decrease in the activity of acetyl CoA carboxylase and citrate cleavage enzyme and an increase in malic enzyme activity occurred at inorganic sulfate levels above (0.42%) and below (0.0002%)

the optimal of 0.02%. However, when methionine supplementation was held constant acetyl CoA carboxylase activity was increased and citrate cleavage enzyme activity was decreased in the livers of rats fed diets containing inorganic sulfate levels above or below the optimal, 0.02%. Under these same dietary conditions malic enzyme activity was increased at the 0.0002% $SO_4^=$ level with no difference occurring at the 0.42% level as compared to the optimal level.

The substitution of cysteine for methionine in the diets previously mentioned resulted in a decrease in the activities of acetyl CoA carboxylase, citrate cleavage enzyme and malic enzyme thus providing additional evidence that cysteine is not the metabolic equivalent of methionine as a dietary supplement.

When diets containing constant levels of total sulfur as sulfate and supplemented with cysteine were fed, acetyl CoA carboxylase activity was decreased and malic enzyme activity was increased at inorganic sulfate levels above or below the optimal. Under the same dietary conditions the activity of citrate cleavage enzyme was found to increase as the level of dietary inorganic sulfate increased. When cysteine supplementation was held constant acetyl CoA carboxylase, citrate cleavage enzyme and malic enzyme activity was decreased in the livers of rats fed diets containing sulfate levels above or below the optimal 0.02%.

The data presented in this investigation show that the level of dietary inorganic sulfate as well as the nature of the neutral sulfur may alter the apparent rate of lipogenesis. These data provide additional evidence of a role for dietary inorganic sulfate as a metabolic regulator.

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INTRODUCTION

The important nutritional role of sulfur compounds in living materials has been well documented. In addition to evidence of the nutritional importance of sulfur containing amino acids, there is evidence that inorganic sulfate is a significant factor in the nutrition of many organisms. Investigations in this laboratory have shown that in order to avoid metabolic stress, inorganic sulfate at a level of 0.02% must be included in the diet of a rat. Recent investigations have demonstrated a role for inorganic sulfate in man.

An increase in the enzyme activity of propionyl CoA carboxylase occurred in rats fed inorganic sulfate at levels below and above the established dietary optimal level of 0.02%. Inorganic sulfate, fed at very low levels resulted in a decrease in the liver level of coenzyme A. An increase in blood glucose levels and liver glycogen, and an increase in the activity of pyruvate carboxylase and phosphoenolpyruvate carboxykinase occurred in rats fed inorganic sulfate at levels above the optimum. Thus, since the level of dietary inorganic sulfate has been shown to influence the glycogenic flux, the level of liver coenzyme A, as well as the activity of certain key enzymes of carbohydrate metabolism, it was postulated that inorganic sulfate may exert an influence on lipogenesis.

Acetyl CoA carboxylase which is considered to be the rate-limiting enzyme of fatty acid synthesisin animal tissue, citrate

cleavage enzyme which is involved in the generation of the carbon precursor, acetyl CoA, for fatty acid synthesis, and malic enzyme which is involved in the generation of the reducing agent, NADPH, for fatty acid synthesis are enyzmes of lipogenesis. Thus these enzymes were chosen as the systems in which to examine whether or not inorganic sulfate influences lipogenesis. The effect of varying the ratio and source of neutral and inorganic sulfur on these enzymes will be determined.

CHAPTER II

REVIEW OF LITERATURE

I. INORGANIC SULFATE

Background

Investigations, previously conducted in this laboratory, have established that the optimum level of dietary inorganic sulfate for the rat is 0.02% (1) and that metabolic alterations occur in rats fed inorganic sulfate at levels below or above the established optimal dietary level (2-9). The following metabolic alterations were observed in rats fed very low levels of dietary inorganic sulfate (0.0002%): a decreased uptake of a test dose of $^{35}SO_4^=$ by rib cartilage mucopolysaccharides (2), a decreased breaking strength of the aorta (3) an impairment of collagen metabolism (4), an enhancement of avitaminosis E[1], an increase in the glycoholic: taurocholic acid ratio in the small intestines (5), a decrease in the level of free coenzyme A in liver microsome preparations,[2] and an increased synthesis of taurine (6) as well as an apparent increased sulfur amino acid requirement (2).

Evidence of other metabolic alterations occurring in rats due to dietary inorganic sulfate supplementation include the finding by

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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, 1970 (abstract).

[2]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.

Roe (7) that inorganic sulfate was an efficient source of sulfate ions for indican formation and the finding of Button et al. (8) that inorganic sulfate was well utilized in the incorporation of sulfate into cartilage mucopolysaccharides. Wellers et al. (9) have shown that dietary inorganic sulfate would supply all the sulfur needs of the adult rat except that required for protein synthesis. Furthermore, White[3] found that there was a significant increase in the enzyme activity of propionyl CoA carboxylase from rats fed inorganic sulfate at the 0.0002% level and the 0.10% and 0.42% level. Thus the activity of propionyl CoA carboxylase was increased in rats fed inorganic sulfate at levels below as well as above the established dietary optimal level of 0.02%. All of these findings demonstrate that in order to avoid metabolic stress, inorganic sulfate must be included in the diet of a rat.

Evidence that inorganic sulfate in the diet of a human can be utilized to replace some of the neutral sulfur was supplied by the finding that dietary inorganic sulfate reversed the negative nitrogen balance in humans who had consumed a semipurified soy diet.[4]

The optimum level of inorganic sulfate for the diet of a rat

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

[4]Zezulkan, A.V. & Calloway, D.H. (1975) Nitrogen balance in young men fed soy protein supplemented with L-methionine, D-methionine, acetyl-methionine, or sulfate. Fed. Proc. *34*, 886 (abstract).

was determined by measuring $^{14}CO_2$ expiration following a test dose of 1-^{14}C-methionine. Rats which were fed diets containing levels of inorganic sulfate above or below 0.02%, experienced an increased expiration of $^{14}CO_2$ indicating an increase in the metabolism of 1-^{14}C-methionine. This investigation was based on the assumption that as dietary sulfate spares methionine the $^{14}CO_2$ expiration would decrease. However, since there was an apparent increase in methionine metabolism in rats fed inorganic sulfate at levels above as well as below 0.02%, there appeared to be a metabolic role for inorganic sulfate other than that of sparing methionine (1). This same experiment was repeated but test meals of U-^{14}C-cysteine were fed. A decrease in $^{14}CO_2$ expiration occurred when levels of inorganic sulfate up to and including 0.42% were fed. The fact that any sparing of cysteine should have been reflected as a sparing of methionine and maximal methionine sparing did not occur at the 0.42% inorganic sulfate level suggested that the higher dietary inorganic sulfate levels influenced the storage of the carbon skeleton of cysteine.[5]

In later investigations using a test meal of U-^{14}C-cysteine, measurements were made of ^{14}C recovered as liver glycogen in rats fed inorganic sulfate at levels above 0.02%. The data demonstrated an increased storage of the carbon skeleton of cysteine as liver glycogen in rats fed inorganic sulfate at levels above 0.02%. These

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[5] Smith, J.T. (1973) Unpublished data.

data indicated that inorganic sulfate has a metabolic role in addition to that of methionine sparing.[6]

Metabolic Role

The level of inorganic sulfate in the diet of a rat has been shown to affect certain enzymes of carbohydrate metabolism. When rats were fed inverse amounts of cysteine and inorganic sulfate in order to keep total sulfur as sulfate constant, an increased activity of phosphoenolpyruvate carboxykinase and pyruvate carboxylase occurred in livers of rats fed increasing amounts of inorganic sulfate (from 0.0002% to 0.42%).[7] A deficiency of inorganic sulfate or cysteine was found to cause an increase in the glycocholic: taurocholic acid ratio as compared to the G:T ratio observed in rats fed diets adequate in cysteine and inorganic sulfate. The same diets which caused an increase in the G:T ratio, caused a decrease in the liver level of free coenzyme A.[8] Other observations have demonstrated an increase in blood glucose levels[9] and liver glycogen[10] in rats fed inorganic sulfate at levels above the optimum. Thus the level of inorganic sulfate in the diet of a rat influences the glycogenic flux, the level of liver coenzyme A, and the activity of certain key enzymes of carbohydrate metabolism.

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

[7] Verma, R., unpublished data.

[8] See footnote 2 page 3.

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

[10] See footnote 5 page 5.

II. ACETYL COENZYME A CARBOXYLASE

Reaction Catalyzed

Acetyl coenzyme A carboxylase [EC 6.4.1.2. Acetyl CoA carbon dioxide ligase (ADP)] catalyzes the biotin-dependent carboxylation of acetyl CoA to form malonyl CoA (10) by the reaction shown in figure 1.

Background

Acetyl CoA carboxylase from avian liver extracts was first identified as one of the two essential enzymatic components (R_1 and R_2) for fatty acid biosynthesis from acetyl CoA (11). The other component, R_2, was later shown to be fatty acid synthetase (12). The finding that fatty acid biosynthesis required bicarbonate (13) led to the discovery that the R_1 fraction catalyzed an ATP-dependent carboxylation of acetyl-CoA (11). It was later demonstrated that the enzymatic carboxylation product was malonyl CoA (14, 15). Wakil et al. (11) observed that the biotin content of carboxylase preparations from avian liver extracts increased during the purification and that the catalytic activity could be blocked by avidin.

In 1952 Brady and Gurin (16) observed that the synthesis of fatty acids from acetate catalyzed by pigeon liver extracts was stimulated by tricarboxylic acids. Following the determination of the enzymatic components and the chemical steps of the system (11-15, 17, 18) further investigations demonstrated that the site of citrate or isocitrate activation is the acetyl CoA carboxylase reaction (19, 20). Vagelos (21) found that an increased sedimentation velocity

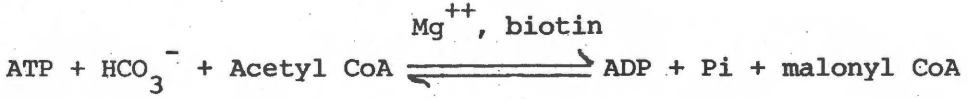

Fig. 1 Reaction catalyzed by acetyl CoA carboxylase.

on sucrose density gradients of the carboxylase accompanied citrate activation. This increased sedimentation velocity was then shown to be due to the polymerization of the protomeric form of the carboxylase (22). Activation by citrate and the polymerization of inactive protomer to give filamentous structures was subsequently shown to be characteristic of acetyl CoA carboxylases of animal origin (23).

Distribution

Acetyl CoA carboxylase was originally shown to be present in extracts of avian liver by Wakil (13) and has been purified from this source (22). In addition it has been purified from bovine adipose tissue (23), rat epididimal adipose tissue (21), rat liver (24, 25), rat mammary gland (26) *Escherichia coli*, (27) brewer's yeast (28), and wheat germ (29). In addition it has been identified in a number of microorganisms (28).

Present evidence indicates that in animal sources acetyl CoA carboxylase is found in the soluble supernatant fraction of homogenized cells (28). However, its association with the microsomal fraction of lactating rabbit mammary gland has been reported (26). Yates et al. (30) appears to have localized the carboxylase to the membranes of the granular cytoplasmic reticulum in hepatocytes of glutaraldehyde-fixed rat liver.

Molecular and Related Properties

Acetyl CoA carboxylases purified from rat and avian liver and bovine adipose tissue exist as enzymatically active polymeric filaments (23). Based on analysis of the rat liver enzyme by dodecylsulfate-polyacrylamide gel electrophoresis, Inoue and Lowenstein (25) concluded that rat liver enzyme contains non-identical subunits with molecular weights of 125,000 and 118,000 daltons. However, Tanabe et al. (24) using [^{14}C] biotin-labelled enzyme preparations obtained evidence which indicated that rat liver acetyl CoA carboxylase has only one kind of subunit which has a molecular weight of 230,000 daltons and contains one molecule of biotin.

The active polymeric form of the avian carboxylase has a molecular weight ranging from 4 to 8 million and is composed of 10 to 20 protomers (22). Both the avian liver and bovine adipose tissue carboxylase upon treatment with 0.5 M NaCl at pH 8-9 have been found to dissociate to the protomer level with the protomeric species having molecular weights of 410,000 ($S^0_{20,W}$=13.1S) (23), and 560,000 daltons ($S^0_{20,W}$=14.7S) (31), respectively.

The structural studies with avian liver enzyme indicate that the identical protomeric units of the carboxylase are composed of four nonidentical subunits which can be resolved into three weight classes 117,000, 130,000, 140,000 daltons with the approximate mass ratio of 2:1:1, respectively. The covalently bound biotinyl group is associated with only one of the 117,000 - dalton subunits (32). On the basis of numbers and types of sites, it has been suggested that the biotin carboxylating site, carboxyl transfer site, citrate

activator site, and biotinyl prosthetic group are located on different subunits within the protomer (10).

In contrast to the animal acetyl CoA carboxylases which undergo purification without loss of structural integrity, acetyl CoA carboxylase from *E. coli* is readily resolved into three essential protein components which retain their characteristic enzymatic activities. These components include: carboxyl carrier protein (CCP-biotin) which is a dimer composed of identical 22,000-dalton subunits; biotin carboxylase which is a dimer composed of apparently identical 51,000-dalton subunits and which carries out the carboxylation of the biotinyl prosthetic group of CCP-biotin; and carboxyl transferase which has an A_2B_2 tetrameric structure composed of 30,000 and 35,000-dalton subunits and which is the catalytic element responsible for the transcarboxylation from CCP-biotin - CO_2 - to acetyl CoA forming malonyl CoA (10, 27).

Substrate Specificity

The apparent substrate affinity constants for acetyl CoA carboxylase isolated from rat liver have been determined. Apparent Km values for acetyl CoA, HCO_3^-, malonyl CoA, Pi, ADP, low ATP concentration, and high ATP concentration have been calculated to be 2.5×10^{-5}, 2.5×10^{-3}, 1.6×10^{-5}, 7.0×10^{-3}, 1.0×10^{-5}, 1.5×10^{-5}, and 4×10^{-5}M, respectively (17, 25, 32). Although maximally active with acetyl CoA, the enzymes from sources other than *E. coli* have been found to carboxylate propionyl CoA almost as well as acetyl CoA. In addition, the enzyme utilizes Mg^{+2} or Mn^{+2} with somewhat different

specificities depending on the enzyme source and the pH of the assay (28).

The enzymes from animal tissues including rat, avian and human liver, bovine and rat adipose tissue, and rat and rabbit mammary gland are activated by tricarboxylic acids (10, 21, 22, 23, 31). Apparent Km values for citrate and DL-isocitrate for avian liver enzymes are both 3-4mM, and 3-4mM and 7-8mM, respectively, for the bovine adipose enzyme (31).

Mechanism of Action

The overall reaction catalyzed by acetyl CoA carboxylases from a variety of animal and microbial sources has been demonstrated to be the result of the two partial reactions shown in figure 2 (1). The participation of the first partial reversible reaction was indicated by the ability of the enzyme to catalyze a Pi, HCO_3^-, and divalent cation-dependent ATP-^{14}C-ADP exchange (20) and an ATP^{32}-Pi exchange (29) dependent upon all the components of reaction (a) but independent of components of reaction (b). The occurrence of a second partially reversible reaction was indicated by the demonstration that acetyl CoA carboxylase catalyzes an avidin-sensitive exchange between malonyl CoA and ^{14}C-labeled acetyl CoA (33) which is dependent upon the components of reaction (b) but independent of the components of reaction (a).

Studies of the *E. coli* carboxylase have shown that only biotin carboxylase and carboxyl carrier protein are required for the first partial reaction and carboxyl transferase and carboxyl carrier protein are required for the second partial reaction (10, 20). Since

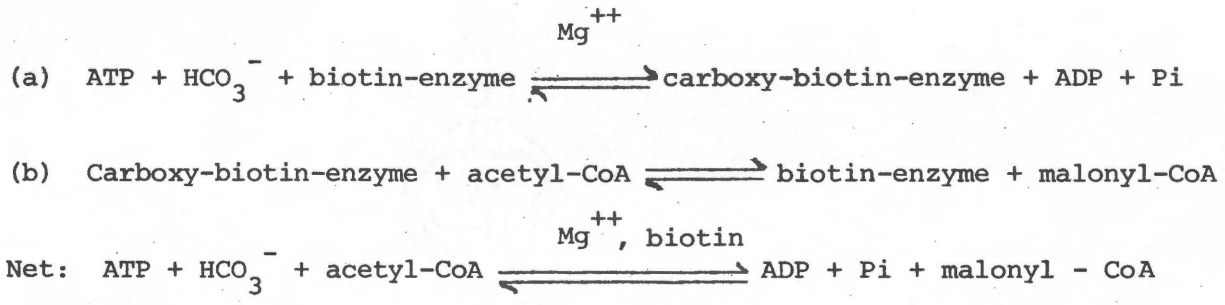

Fig. 2 Acetyl CoA Carboxylase reaction sequence.

studies have shown that the catalytic sites for the two partial reactions reside on different subunits, distinct from the carboxyl carrier protein, and since the biotinyl group is attached to the apo-carboxyl carrier protein through its side-chain (maximal length 14Å) to a lysyl *e*-amino group, the biotinyl group should be capable of oscillating between the carboxylation and transcarboxylation sites on biotin carboxylase and carboxyl transferase (34) as shown in figure 3. Kinetic evidence obtained by Numa et al. (35) indicates that the reaction proceeds via a "bi bi uni uni Ping-Pong" mechanism in which following the formation of carboxybiotin, ATP binds prior to HCO_3^- with Pi release subsequent to ADP followed by the binding of acetyl CoA and the release of malonyl CoA.

Kaziro et al. (36) demonstrated that one ^{18}O atom from ^{18}O-labeled HCO_3^- is incorporated into orthophosphate for every two atoms of ^{18}O incorporated into the carboxyl of the carboxylated biotin prosthetic group of propionyl CoA-carboxylase. As in the reaction catalyzed by propionyl-CoA carboxylase, bicarbonate has been found to be the active species in the carboxylation of free-biotin catalyzed by the biotin carboxylase component from *E. coli* and avian liver (10). It has been suggested that the binding of CO_2 to the biotin-enzyme complex occurs by the concerted mechanism (36) shown in figure 4, a nucleophilic attack of the ureido ring of biotin at an electrophilic center of the bicarbonate followed by an attack of the bicarbonate oxygen on the terminal phosphate of ATP. An alternative mechanism (fig. 5) involving carbonic-phosphoric anhydride as a tightly bound enzyme intermediate has been proposed (37).

Although the carboxylation of acetyl CoA has been found to

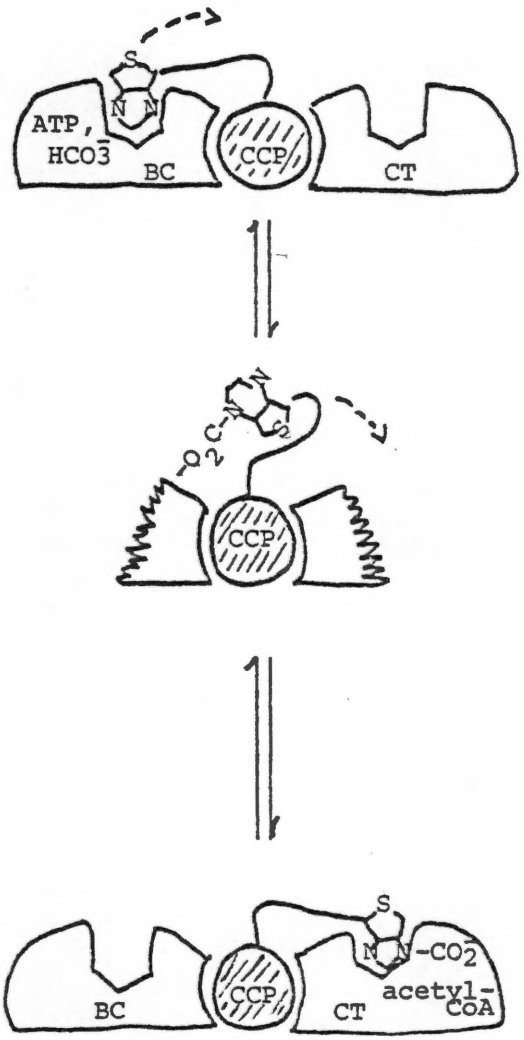

Fig. 3 Model for intersubunit translocation of carboxybiotin of acetyl CoA carboxylase. BC, biotin carboxylase; CCP, carboxyl carrier protein; CT, carboxyl transferase (34).

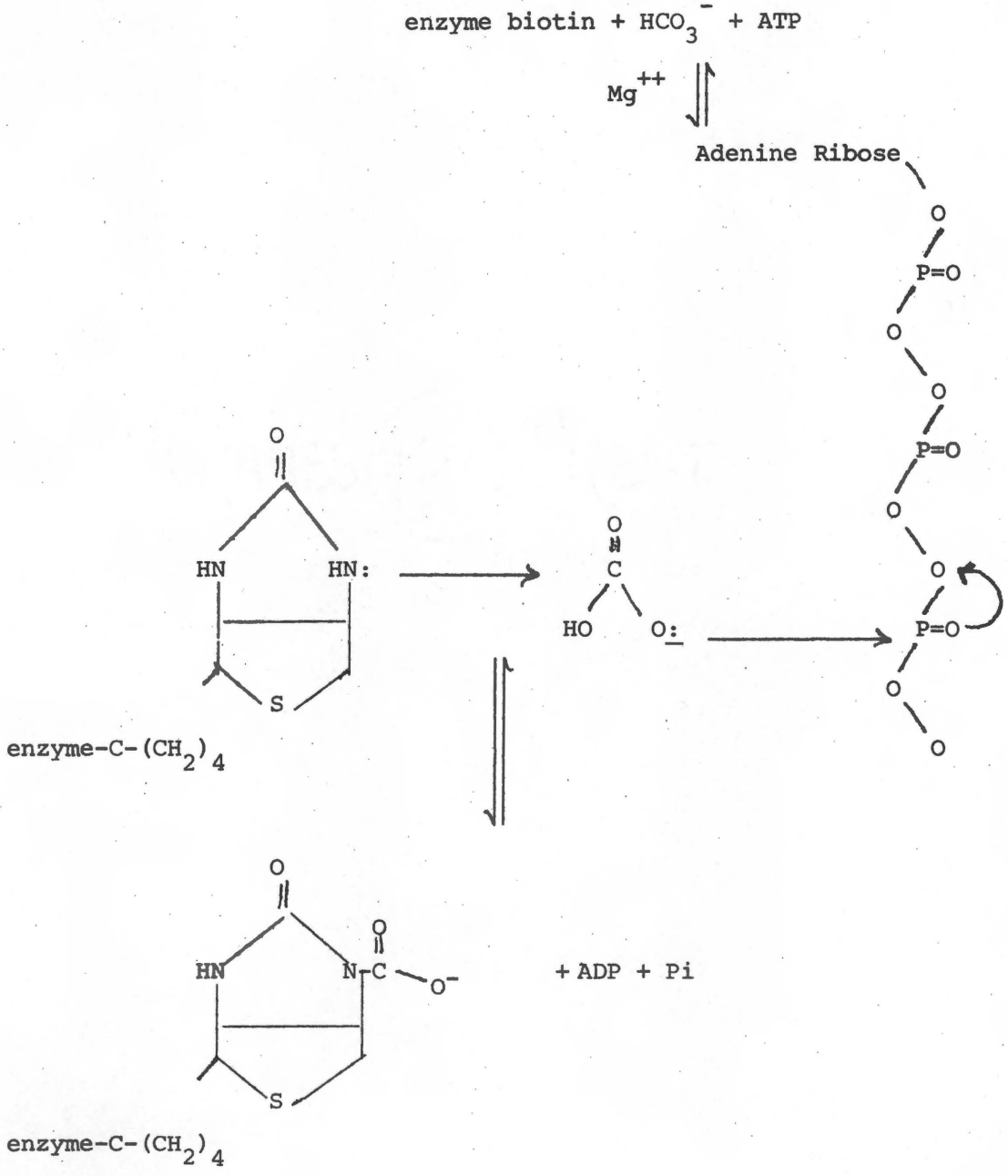

Fig. 4 Suggested mechanism for the carboxylation of the biotin enzyme complex (36).

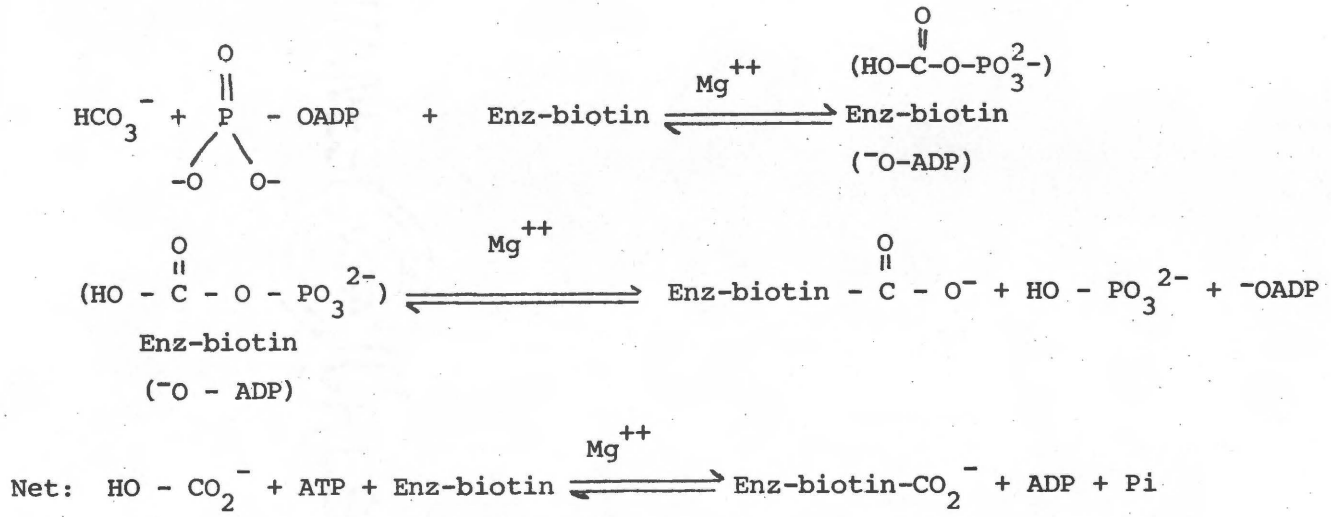

Fig. 5 Suggested mechanism for the carboxylation of the biotin enzyme complex involving carbonic-phosphoric anhydride intermediate (37).

require nucleophilic activation at the α-carbon atom, the mechanism of this activation is not known. Proton release from the α-position of the acyl-CoA has been shown to require the presence of the carboxylated biotinyl prosthetic group indicating that the process is concerted or carboxylation of the prosthetic group causes a conformational change in the enzyme which facilitates proton removal (34).

Enzyme Activation and Inhibition

Using the two exchange reactions of ATP-^{32}Pi exchange and malonyl CoA-acetyl -^{14}C-CoA exchange Ryder et al. (38) demonstrated that citrate activated both partial reactions catalyzed by rat liver acetyl CoA carboxylase. Studies with model partial reactions, that is, the carboxylation of free biotin and the carboxylation of acetyl pantetheine (an analog of acetyl CoA) have also shown that both partial reactions are citrate-activated (39).

In addition to citrate and isocitrate which are almost equally effective activators, fluorocitrate (21) and (-)-hydroxycitrate (10) have been found to activate the enzyme. However, tricarballylate, (22) a tricarboxylic acid analog of citrate and isocitrate, and certain dicarboxylic acids including malonate, methylmalonate, and malate (10) have been found to behave as apparent activators since they retard the transition of the active to the inactive species rather than promoting the reverse transition.

Studies of Numa et al. (40) have shown rat liver carboxylase to be reversibly inactivated by low temperature (0°) and reactivated by preliminary incubation with tricarboxylic acid activator at 25°

prior to assay. Activation was also found to be achieved by preliminary incubation at 37° with Mg^{++} but with the addition of citrate to the subsequent assay (41). Thus, two conformational changes, (fig. 6) a slow temperature-dependent transition requiring Mg^{++} or citrate and a rapid citrate-dependent transition, may be involved in the activation of rat liver enzyme (20).

As stated previously, the shifting of the protomer-polymer equilibium toward the active state by citrate activation is indicated by the finding that citrate induces a change in the sedimentation coefficient of the carboxylase accompanied by a transition from protomeric to active filamentous form (21, 22). Gregolin et al. (22) investigated the factors affecting the reversible inter-conversion between the protomeric and polymeric forms of the avian enzyme and found that certain anions (citrate, isocitrate, malonate, tricarballylate, sulfate, Pi) or acetyl CoA, high protein concentration, and pH 6 to 7 promote aggregation of the protomer whereas carboxylation of the enzyme, Cl^{-}, and pH values greater than 7.5 cause dissociation of the polymeric form. Under conditions for carboxylase assay, it was shown that carboxylation of the enzyme promotes dissociation with citrate or isocitrate but not tri-carballylate, Pi or malonate, can prevent. Furthermore, since the enzyme would exist in its carboxylated form in the presence of ATP plus Mg^{++} or malonyl CoA, these agents cause dissociation which can be prevented by acetyl CoA or ADP. Thus, the introduction of a carboxylate at position 1'N of the biotinyl prosthetic group apparently produces a strained configuration such that only true

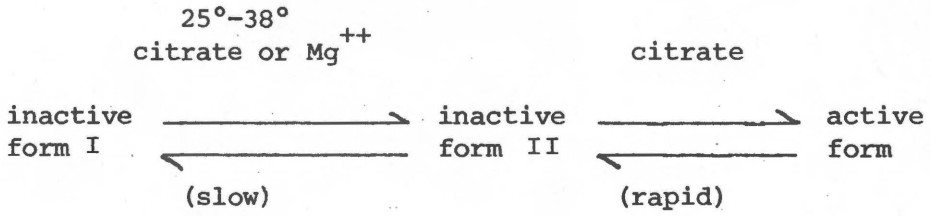

Fig. 6 Two possible conformational changes involved in rat liver enzyme activation.

activators are able to constrain the enzyme and prevent its depolymerization (22).

Malonyl CoA as well as fatty acyl CoA derivatives, such as palmitoyl CoA, stearoyl CoA, and oleoyl CoA are competitive inhibitors with respect to citrate. These derivatives promote depolymerization of the carboxylase, which can be prevented by citrate. In addition several hypolipidemic drugs (2-methyl-2-phenoxypropionate derivatives) have been found to inhibit the carboxylase by reversing the activator-promoted aggregation of the carboxylase (10).

Initial investigations of the mechanism of tricarboxylic acid activation revealed that the Km values for the substrates were not materially affected by activation. Since activation increases the reaction rate of bound substrate, rather than altering the affinity of the enzyme for the substrates, citrate has been classified as a positive allosteric effector of the Vmax type (22, 33).

Studies on the inactivation of rat liver enzyme by avidin, the specific biotin-binding protein from egg white, have shown that the biotin prosthetic group of the citrate activated enzyme was completely inaccessible to avidin whereas in the absence of citrate the biotinyl group was accessible to avidin. In experiments in which the rates of inactivation were increased by raising the avidin to carboxylase ratio, acetyl CoA was shown to act synergistically with

activator. These findings indicate that the biotinyl prosthetic group may become shielded by neighboring groups as a result of conformational changes at the active site induced by citrate (38).

A more recent explanation of the citrate effect is that the carboxybiotin prosthetic group may be brought into closer proximity to substrate binding sites by conformational changes at the active site induced by citrate and that citrate may enhance the affinity of a binding site in the vicinity of the substrate for the ureido ring of the prosthetic group and thus facilitate reaction (20).

3'5'-cyclic adenosine monophosphate (cAMP) or its N^6, $O^{2'}$-dibutyryl derivative was found to inhibit the incorporation of acetate -^{14}C or glucose -^{14}C into hepatic fatty acids both with liver slices *in vitro* (10) and *in vivo* (42). The finding that dibutyryl 3',5'-cAMP also inhibits the incorporation of tritium from tritiated water into fatty acids indicates that the effect is due to a decreased rate of *de novo* fatty acid synthesis, rather than to a dilution of ^{14}C labeled intermediates of the pathway caused by cAMP-activated glycogenolysis (43).

Carlson and Kim (44) found that a partially purified preparation of the rat carboxylase was inactivated by ATP in a time-and temperature dependent reaction, these findings being independent of cAMP. Moreover, when carboxylase inactivated by exposure to [^{32}P] ATP was precipitated with antibody, isotope incorporation into the precipitate paralleled enzyme inactivation and immuno-precipitable ^{32}P label was located in phosphoserine and phosphothreonine residues.

Furthermore, it was found that dephosphorylation of labelled enzyme accompanied magnesium - dependent activation. These data suggest that acetyl-CoA carboxylase may be regulated by a phosphorylation-dephosphorylation mechanism.

III. CITRATE CLEAVAGE ENZYME

Reaction Catalyzed

ATP citrate lyase [EC 4.1.3.8, ADP: citrate oxalacetate-lyase (CO-A-acetylating and ATP-dephosphorylation); also known as the citrate cleavage enzyme] catalyzes the reations shown in figure 7. (45).

Background

In 1946 Lipton and Barron (46) reported that in mammalian brain homogenates, acetylcholine was formed from citrate, ATP, choline, and coenzyme A and they attributed this synthesis to the reverse of citrate synthetase. However, the ATP requirement suggests that the synthesis which they studied took place by way of citrate cleavage enzyme and choline acetylase (45). Citrate cleavage enzyme was first found in pigeon liver in 1953 by Srere and Lipman (47). The enzyme was partially purified from pigeon liver (47) and later was more extensively purified from chicken liver (48).

Citrate cleavage enzyme catalyzes one of the most complex enzyme reactions in that the reaction has three substrates, four products, and a metal ion requirement (45). Srere was able to demonstrate that a single enzyme catalyzed the entire process and later established the stoichiometry of the reaction (49). The enzyme

Fig. 7 Reaction catalyzed by citrate cleavage enzyme.

was crystalized from rat liver by Inoue et al. (50). These crystals were shown as cubic in form. Later the crystalline enzyme isolated from rat liver was shown mainly as an amorphous material referred to as flat plates and its homogeneity was established by analytical centrifugation, moving boundary electrophoresis, and immunological analysis (51). A series of studies on enzyme mechanism have been carried out on rat liver enzyme by Inoue and his coworkers (52-56) and most of these studies have been confirmed and extended by Srere and coworkers (55, 56) as well as other investigators (57).

Distribution

Citrate cleavage enzyme is widely distributed in animal tissues with especially high levels being found in liver, adipose tissue, lactating mammary gland, adrenals, and brain (45). The enzyme has been found in pigeon liver (47), beef brain and pig heart, certain bacteria (48) and several tissues of rat, rabbit, and chicken (45). The enzyme has been localized in the cytosol compartment of the cell (45).

Molecular and Related Properties

The molecular weight of rat liver citrate cleavage enzyme reported by Inoue et al. (50) was 500,000 daltons based on a diffusion coefficient 2.62×10^{-7} cm^2/ sec and a sedimentation coefficient of 13.5S. An average of the molecular weight of rat liver enzyme determined by several different methods was calculated to be 400,000 $\pm$ 40,000. Viscosity determinations in 7M guanidine HCl indicated a subunit weight of 42,500 daltons. These data as well as data from amino acid analysis of rat liver enzyme using a tryptic digest

correspond to between 8 and 10 subunits (45).

Substrate Specificity

The apparent substrate affinity constants for citrate cleavage enzyme isolated from rat liver have been determined. The Km values for MgATP, Mg citrate, CoA, MgADP, Pi, oxalacetate, and acetyl CoA are calculated to be 280, 70, 1.6, 4, 1.5, 180 and 10 uM, respectively (45, 57).

There is evidence that the kinetic behavior of the enzyme in the presence of citrate depends strongly on the Cl^- concentration. Using the method of Srere, where the reaction is coupled to the malate dehydrogenase reaction, Plowman and Cleland (57) demonstrated non Michaelis-Menten kinetics at low Cl^- concentration, but when Cl^- was 0.25M, then citrate followed almost normal kinetics.

The enzyme from rat and chicken liver was found to be highly specific for citrate with isocitrate, cis-aconitate, acetate, malonate, succinate, and malate being inactive in the standard hydroxamate assay (47, 49, 50, 58). Although tricarballylate cannot yield any cleavage products, it is active in the assay (58). In addition, the enzyme is specific for ATP with CTP, GTP, UTP, and ITP exhibiting little, if any activity (45, 50). The most efficient divalent metal cation is Mg^{++}. Manganous and cobalt ions are each approximately 60% to 80% as active as Mg^{++} (49), and the following cations are inactive: Ni^{++}, Fe^{++}, Fe^{+++}, Cu^{++}, and Zn^{++} (45). Furthermore, Srere (49) has reported that coenzyme A cannot be replaced by pantetheine.

Mechanism of Action

A series of studies on enzyme mechanism have been made using rat liver citrate cleavage enzyme. From these studies a reaction scheme for the citrate cleavage enzyme reaction has been proposed. This scheme has been represented as an ordered sequence of partial reactions shown in figure 8 (45, 59). The first step of the reaction (a) involves phosphorylation of the enzyme. Enzyme phosphorylation is indicated by the finding that incubation of the enzyme with ^{32}P-ATP in the presence of Mg^{++}, but in the absence of citrate and CoA, followed by gel filtration results in labeling of the recovered enzyme, whereas incubation with 8-^{14}C-ATP yields unlabeled enzyme (53). Indications are that there is an incorporation of not less than 2 moles of ^{32}P per mole of enzyme (56).

The nature of the chemical linkage of phosphate to enzyme is not clear. The finding that alkaline hydrolysis of phosphoenzyme -^{32}P results in a 50% yield of phosphohistidine -^{32}P has led to the suggestion that a phosphohistidine exists on the enzyme (56). The phosphorylated enzyme (EP) has also been shown to have a U-shaped pH profile which is common to acyl phosphates. In addition, hydroxylamine has been found to cause a rapid quantitive release of phosphate from the enzyme phosphate. These findings have led to the suggestion that an acyl phosphate, not a phosphohistidine, exists on the enzyme (60). Das et al. (56) have shown that hydrolysis of enzyme phosphate is slow and follows partial digestion of the enzyme. Srere (45) has suggested that amino groups released during hydrolysis may catalyze a breakdown of phosphohistidine. In addition, Spector (59) has

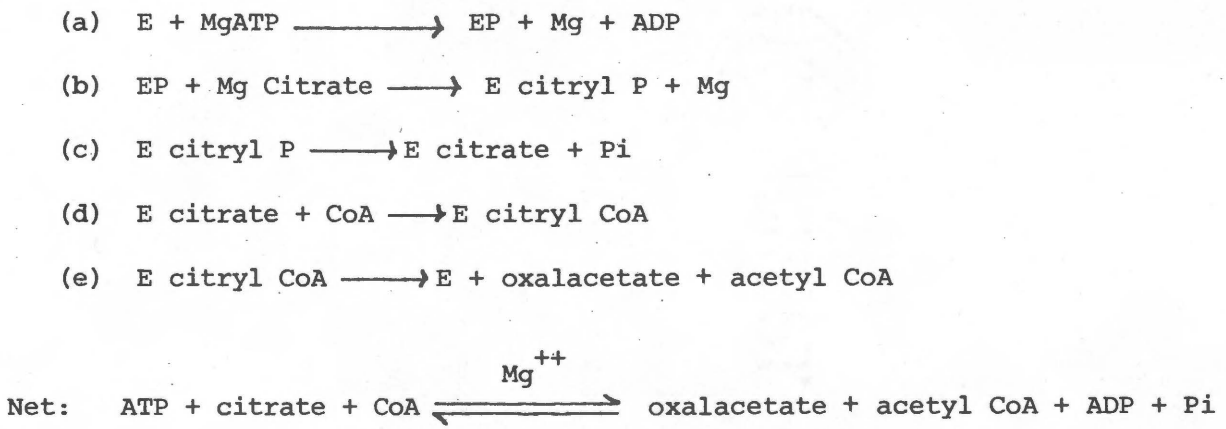

Fig. 8 Reaction scheme catalyzed by citrate cleavage enzyme.

suggested a possible envolvement of both a histidine residue and a carboxyl group in phosphoryl transfer at the active site of the enzyme.

The second step (b) in the reaction sequence involves a reaction of phosphoenzyme with citrate in the presence of Mg^{++} to form enzyme-bound citryl phosphate. Chemically synthesized citryl phosphate has a U shaped pH- stability profile, with maximum stability at pH 8.5-9.5 (61). In the enzymatic reaction with CoA, Walsh and Spector (61) report the Michaelis constant of citryl phosphate to be 2500 uM.

The enzyme-bound citryl phosphate reacts further (reaction c) to yield citrylated enzyme (53). Inoue et al. (53) found that incubation of citrate cleavage enzyme with ATP, Mg^{++}, and labeled citrate but with CoA omitted, followed by gel filtration, yields a citrylated enzyme. In addition, they found that treatment of the isolated citrylated enzyme with CoA resulted in the formation of oxalacetate and acetyl CoA. These findings support the possibility of the citryl enzyme being an intermediary in the reaction sequence.

The next step (d) in the reaction scheme shows a reaction of the citryl enzyme with CoA to form an enzyme-bound citryl CoA. Consistent with this proposed step is the finding that chemically synthesized citryl CoA will yield acetyl CoA and oxalacetate in the presence of the citrate cleavage enzyme. The reaction does not require ATP or Mg^{++} and proceeds to the extent of approximately 50% indicating that only one optical isomer of citryl CoA is recognized by the enzyme (54).

An alternate proposal for this step is that the citryl enzyme cleaves citrate to form enzyme acetate and oxalacetate and that enzyme acetate subsequently reacts with CoA to yield acetyl CoA (59).

However, the fact that tricarballylate which cannot undergo cleavage and which when incubated with enzyme, ATP, Mg^{++}, and CoA will form tricarballyl - CoA indicates that the above proposal is unlikely (62).

The last step (e) of the reaction sequence involves the cleavage of enzyme-bound citryl CoA to yield enzyme, acetyl CoA, and oxalacetate. Although the enzymatic cleavage of citrate has been found to be reversible (5% of that of the forward reaction), the overall reaction is favorable to the cleavage of citrate (53). Spector (59) has suggested that the favored forward direction of the reaction may be related to the preservation of the energy of the phosphate anhydride bond of ATP through each phase of the reaction sequence.

Enzyme Activation and Inhibition

As previously mentioned a divalent metal cation is required for enzymatic activity. There is evidence that citrate may activate the enzyme since it was found that citrate activates the enzymatic-hydrolysis of citryl CoA (63).

By titrating the enzyme with DTNB [5,5' dithio bis(2-nitro-benzoate)] in urea or sodium dodecyl sulfate, Cottam and Srere (64) have found that the total sulfhydryl content of purified rat liver enzyme was 45 to 60 moles per mole (500,000g) of enzyme. They also demonstrated that the enzyme loses a majority of its activity when 6 to 8 of its sulfhydryl groups are reacted with DTNB and that these sulfhydryl groups were partially protected by Mg citrate, but not by Mg ATP. These findings indicate that the sulfhydryl groups may be located at the active site of the enzyme. In addition, Cottam and Srere (64) found that the enzyme is inhibited 50% by arsenite, and is

also inhibited by oxidized glutathione, oxidized BAL (British anti-Lewisite preparation), and lipoate. The inhibition of disulfide was found to be pH dependent with maximal inhibition at pH 8.7 and virtually no inhibition at pH 7.4. Thus, since a large part of the instability of the enzyme activity is due to easily oxidized sulfhydryl groups, partial recovery of the activity can be achieved by incubation of the enzyme with a sulfhydryl compound.

Inoue et al. (51) have shown that orthophosphate at a concentration of 2×10^{-2}M causes a 70% inhibition of the enzyme. They also demonstrated that the enzyme is inhibited by ADP and this inhibition is competitive with respect to ATP. Atkinson (65,66) has proposed that cleavage enzyme activity is a sensitive function of the "energy charge" of the adenylate system (ATP + 1/2 ADP)/(ATP + ADP + ATP).

Srere (45) has reported that the enzyme is inhibited by pyridoxal and its analogs. Watson et al.(58) have observed that (-)-hydroxycitrate exerts a steroselective inhibition of citrate cleavage enzyme and that (+) - allohydroxycitrate was not inhibitory.

IV. MALIC ENZYME

Reaction Catalyzed

Several names have been given the enzyme which catalyzes the oxidative decarboxylation of L-malate to pyruvate and CO_2 including malate dehydrogenase: NADP-linked, malate dehydrogenase-decarboxylating, [L-malate: NADP - Oxidoreductase (decarboxylating) EC 1.1.1.40] and malic enzyme (67). Malic enzyme catalyzes the reaction shown in figure 9, (reaction 1). Pigeon liver preparations of this enzyme also possess oxalacetate decarboxylase (reaction 2) and pyruvate reductase activities (reaction 3) and convert oxalacetate to L-malate in the presence of NADPH and L-malate to lactate in the presence of NADP (reactions 4 and 5) (68).

Background

In 1947 Ochoa et al. (69) were the first to describe the enzymatic oxidative decarboxylation of L-malate to pyruvate and CO_2 in pigeon liver extracts and to show that the enzyme was found in the cytosol fraction of the extract. They subsequently suggested the name "malic enzyme" to denote this particular enzyme. The enzyme from pigeon liver was initially purified by Rutter and Lardy (70) and was crystallized by Hsu and Lardy (71). Simpson and Estabrook (72) observed the presence of malic enzyme in both the cytosol and mitochondria isolated from bovine adrenal cortex preparations. Subsequently, a screening of several animal species led Brdiczka et al. (73) to conclude that the subcellular distribution of malic enzyme is both species and tissue specific.

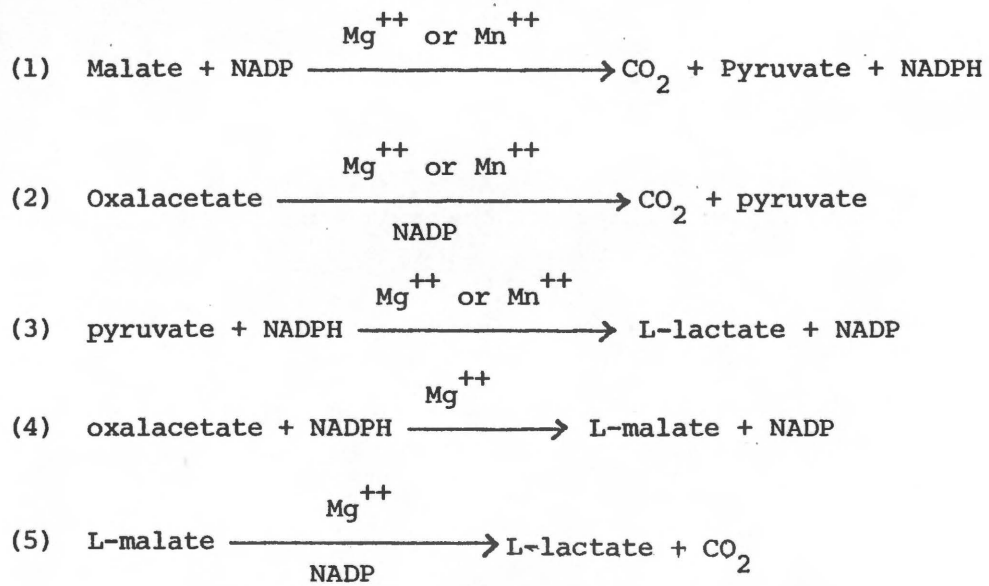

Fig. 9 Reactions catalyzed by malic enzyme.

Shortly after the discovery of malic enzyme a physiological role for the enzyme was postulated by Ochoa et al. (69). They proposed that malic enzyme could participate in CO_2 fixation forming a C_4 intermediate and thus participate in the process of gluconeogenesis. However, the discovery of Utter et al. (74) of the presence of pyruvate carboxylase in liver mitochondria suggested the possible participation of this enzyme in gluconeogenesis and led to a reevaluation of the role of malic enzyme. Shrago et al (75) found that during conditions favoring gluconeogenesis such as starvation, there was an increase of gluconeogenic enzymes but no measurable increase in malic enzyme. Later, observations by Wise and Ball (76) of a decrease in malic enzyme during starvation followed by a marked overshoot of the enzyme upon refeeding a low fat, high carbohydrate diet and a parallel increase in activities of glucose -6 - phosphate dehydrogenase and 6 - phosphogluconate dehydrogenase were made. Since a product of the action of these enzymes is NADPH and there was a positive correlation between hepatic lipogenesis and malic enzyme activity, it was concluded that the true role of this enzyme was in lipogenesis. Although there is a correlation between malic enzyme and lipogenesis in most studies, there have been observations which have suggested other roles for malic enzyme. In 18-day-old rat fetuses, the rate of hepatic fatty acid synthesis was observed to parallel the rate of activity of citrate cleavage enzyme while there was no detectable malic enzyme in fetal liver (77). In addition, the induction of rat liver malic enzyme by restriction of dietary protein was studied in conjunction with the biosynthesis of fatty acids and the activities of fatty acid synthetase, citrate cleavage enzyme, glucose 6-PO_4 dehydrogenase, and glutathione reductase (78). Under

the condition of protein restriction Stark et al.(78) found a positive correlation between malic enzyme activity and glutathione reductase but no detectable correlation between malic enzyme activity and lipogenesis indicating a possible role for malic enzyme in furnishing reducing equivalents required for the formation of reduced glutathione.

Distribution

Malic enzyme has been investigated in heart, brain and liver of rat and of chicken; pigeon liver; pig heart; cod and rabbit testes; and muscle and mouse erythrocytes as well as a variety of micro-organisms and plants (67).

The intracellular localization of malic enzyme in animal cells was described in a review by Frenkel (67). Malic enzyme has been found in both the cytosol and mitochondria of several different tissues and in several different species. Varients in the mitochondria and cytosol have been separated and studied in rat, pig, and bovine hearts as well as bovine brains and adrenal cortex. In these studies it has been observed that the properties of the enzyme obtained from one compartment are considerably different from those of the other compartment (73).

The rat liver enzyme appears to be almost exclusively localized in the cytosol with less than 2% of the total enzymatic activity associated with the mitochondrial fraction (67). Rat liver malic enzyme was resolved by disc electrophoresis into 3 isozymes which were found to be present in each state of isolation indicating that they are stable molecular entities. Using an antibody prepared against

rat liver malic enzyme, extracts of all rat tissues studied (adipose tissue, adrenal, testis, kidney, and heart) were identical in terms of immunological properties. Furthermore, these same tissues were identical in terms of electrophoretic mobility except cardiac tissue contained an additional more rapidly migrating isozyme which may be located in the mitochondria (79). The presence of two different types of cytosol malic enzyme in rat tissues: type A (adipose tissue) and type H (heart) has been suggested. In addition, the presence of both types in liver cytosol has been postulated (80).

Molecular Structure and Related Properties

Malic enzyme has been crystallized and extensively investigated from pigeon and rat liver preparations. The enzyme is homogenous throughout ultracentrifugation and gradient chromatography. Crystalline pigeon liver malic enzyme has a sedimentation coefficient of 10.0S, an apparent partial specific volume of 0.74 and an apparent diffusion coefficient of 3.17×10^{-7} cm^2 per sec and a molecular weight of 2.8×10^5 (71). From the molecular weight, the number of NADP-binding sites was estimated to be 4 (81). Based on the estimation of NADP-binding sites and sedimentation constants of the products of alkaline dissociation in purified pigeon liver enzyme, Hsu and Lardy (81) suggested that malic enzyme possesses a tetrameric structure.

Since inactivation of the enzyme upon storage is reversed by incubation with dithiothreitol, the enzyme is believed to contain a a sulfhydryl group at or near the active center (71). The molecular weight of rat liver malic enzyme was calculated from high speed

sedimentation equilibrium studies to be 268,000 daltons (75). The sedimentation coefficient ($S_{20,W}$) of rat liver enzyme was found to be 10.2. From studies of *in vitro* interspecies hybridization of partially purified rat and hamster liver malic enzyme, the major isozyme in each species has been found to be an isologous tetramer with a subunit molecular weight of 67,000 daltons (82).

Substrate Specificity

Reactions catalyzed by pigeon liver malic enzyme require either NADP or NADPH and a divalent metal ion with Mn^{++} preferred (67). Hsu et al (83) have calculated the Michaelis constants for NADP and malate, at pH7, and found them to be 1.4uM and 86uM, respectively. The apparent Michaelis constants for the back reaction were found to be 13mM, 6.4mM and 2.1uM for bicarbonate, pyruvate and NADPH, respectively. Since the limiting Km for substrates in the forward direction are equal to or slightly below concentrations found in the cell and since the Km for pyruvate is two to three orders of magnitude higher than the concentration found in the cell, it has been suggested that the forward reaction is the only physiologically significant reaction in mammalian systems (83).

Mechanism of Action

Hsu and Lardy (71) have postulated a mechanism for malic enzyme shown in figure 10. Due to the ability of malic enzyme to use oxalacetate as a substrate in both decarboxylation and reduction reactions, the authors have suggested that this compound acts as an enzyme bound intermediate during catalysis. From kinetic analysis of reaction 1 (fig. 9) a sequential mechanism involving the addition of NADP

followed by malate and release of the products in the order of CO_2, pyruvate, and NADPH was proposed. This ordered sequence is shown in figure 10, steps 1-6. Oxalacetate decarboxylase activity can be accounted for by the formation of a central enzyme NADPH-oxalacetate ternary complex from the required substrate (step 7), which undergoes decarboxylation forming enzyme - NADPH - pyruvate complex (step 4) followed by the release of pyruvate and NADPH (steps 5 and 6). In the case of the oxalacetate reduction activity, there is the formation of the oxalacetate enzyme intermediate (step 7), which then isomerizes to an enzyme - NADP - malate complex (step 3), followed by release of malate and NADP (steps 2 and 1) (68).

More recently, Simerlik[11] has proposed a mechanism for malic enzyme in which hydride transfer is accompanied by the transfer of a proton to an active site residue with a pK of 5.4 followed by decarboxylation and the release of CO_2. Subsequently, enol pyruvate is protonated by a proton from water which is coordinated with the metal.

Enzyme Activation and Inhibition

Hsu and Lardy (71) have shown that all five activities shown in figure 9. for pigeon liver malic enzyme have requirements for a divalent metal and for NADP in its oxidized (malic enzyme and oxalacetate decarboxylase) or reduced form (pyruvate and oxalacetate). Rutter

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[11]Simerlik, M.I. (1975) Kinetic studies on malic enzyme. Dissertation abstract Int. B. (1976) *36*, 5013.

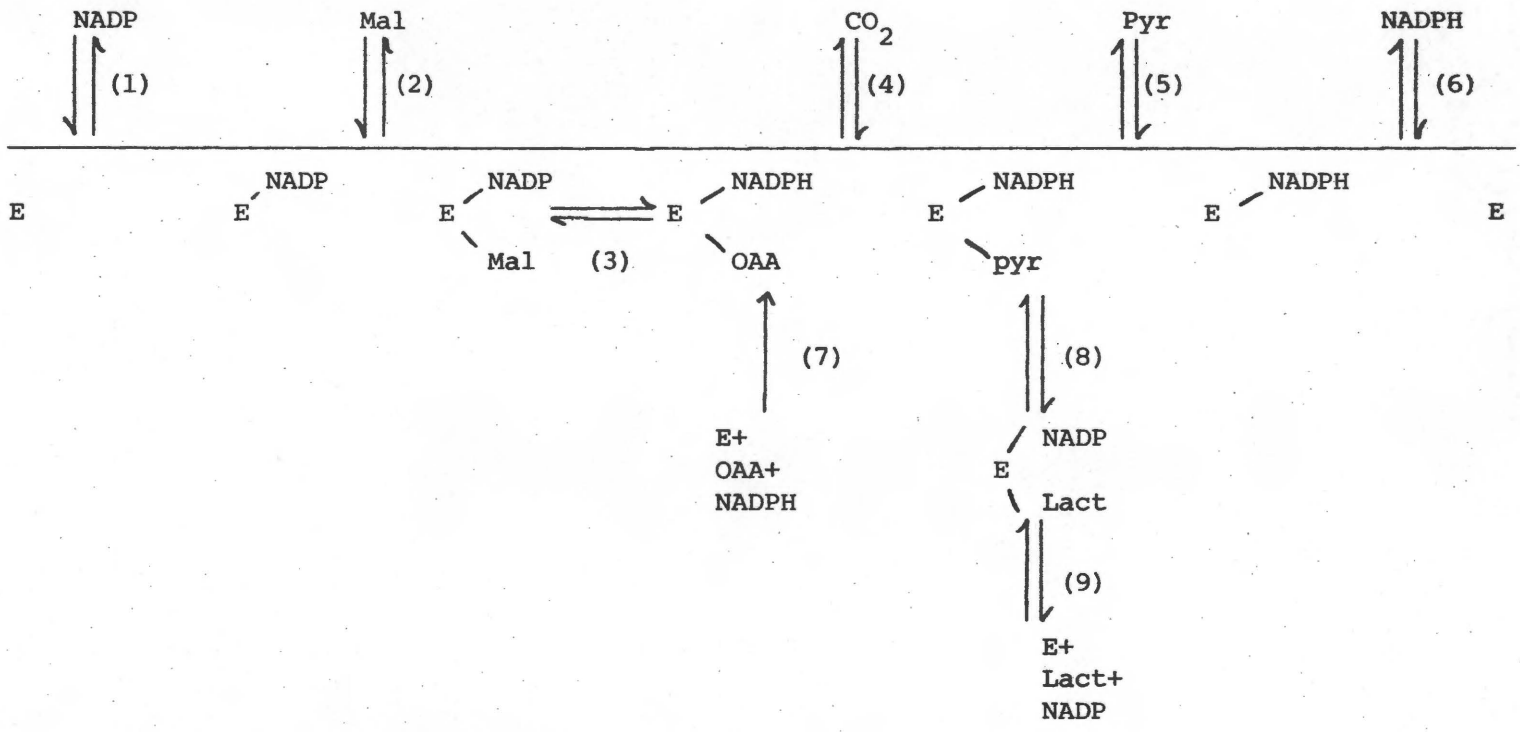

Fig. 10 Reaction Scheme Catalyzed by Malic Enzyme. E, metal enzyme; Mal, L-malate; Pyr, pyruvate; Lact, L-lactate; OAA, oxalacetate (71).

and Lardy (70) have demonstrated that several metal ions in addition to Mg^{++} and Mn^{++} promote the oxidative decarboxylation of malate. They found that Co^{++} in high concentrations allowed a higher initial velocity than Mn^{++} and that Cu^{++} was weakly effective. In addition, Hg^{++} was found to be a strong inhibitor of the reaction.

Oxalacetate has been shown to be a competitive inhibitor of the oxidative decarboxylation of L-malate (84). 5-5'- dithio bis(2-nitrobenzoic acid) was shown to induce a reversible loss of malic enzyme activity and an increase in pyruvate reductase activity, while the oxalacetate reductase activity remained constant. These findings are compatible with the proposed mechanism shown in figure 10 in which a blockage of the CO_2 site by modification of the sulfhydryl groups should lead to the loss of activities involving decarboxylation such as malate decarboxylase (68).

V. LONG TERM REGULATION OF ACETYL CoA CARBOXYLASE, CITRATE CLEAVAGE ENZYME, AND MALIC ENZYME

The activities of acetyl CoA carboxylase, citrate cleavage enzyme, malic enzyme as well as the activity of other lipogenic enzymes undergo relatively long-term regulatory changes secondary to several factors. The factors involved in the long-term regulation of lipogenesis in animal tissue have been reviewed (63, 85-87). Some of the factors which have been studied extensively include nutritional, genetic, hormonal, and developmental.

Nutritional Factors

As a result of fasting, a rapid decline in fatty acid synthesis as well as in the level of activity of acetyl CoA carboxylase (35), citrate cleavage enzyme (88), malic enzyme (76), and other key lipogenic enzymes has been observed (85, 89). After refeeding a high-carbohydrate diet, a rapid stimulation of fatty acid synthesis and a marked and coordinate rise in the activity levels of all the key enzymes of lipogenesis has been noted (35, 63, 85-88). Fat-feeding has been found to result in a reduction of lipogenic enzyme activity levels (86, 89) which is consistent with the finding that the increase in activity levels after refeeding was more marked on a fat-free diet than on a balanced diet (86).

From the result of these and similar studies it has been concluded that changes in fatty acid synthesis is usually coordinated with alterations in the enzymes of lipogenesis. However, these studies of the coordinated fluctuation of the activity level of lipogenic

enzymes do not distinguish between an effector-mediated response and an altered enzyme concentration. Thus, investigations have been made to determine whether some of these nutritional effects are secondary to changes in enzyme content or to changes in the catalytic efficency of the enzymes (85). Acetyl CoA carboxylase antibody, which will inactivate and precipitate both protomeric and polymeric forms of the enzyme was used in immunochemical titrations of liver extracts derived from fasted, fat-free refed, diabetic and chow fed rats. Increasing amounts of specific enzyme activity from each of the four extracts were added to a constant amount of antibody. Following immunochemical precipitation, carboxylase activity was measured in the supernatant. Although the level of carboxylase activity decreased in starvation and diabetes and increased following refeeding, the equivalence points were identical for all four types of liver extracts when based on the amount of enzyme activity added. Therefore, the differences in specific activity reflected differences in enzyme content (90).

To determine whether these differences in acetyl CoA carboxylase content reflected changes in enzyme synthesis, the extent of [^{3}H] leucine incorporation into protein precipitated by antiacetyl CoA carboxylase following pulse labeling was employed. It was found that changes in enzyme synthesis quantitatively accounted for the changes in enzyme levels for all states except fasting (91). Gibson et al. (92) used similar techniques to demonstrate that increased enzyme synthesis was responsible for the rise in activities of citrate cleavage enzyme and malic enzyme upon refeeding fasted animals.

Actinomycin D and puromycin, which inhibit transcription and translocation, respectively, were found to block the anticipated rise in acetyl CoA carboxylase and fatty acid synthetase activities in response to refeeding starved rats. Similarly, the adaptive rise in citrate cleavage enzyme was found to be blocked by the administration of puromycin prior to refeeding (92). Furthermore, the insulin-stimulated increase of fatty acid synthesis in the alloxan-diabetic rat was found to be inhibited by concurrent administration of actinomycin D (93). Thus, these results indicated that the change in the enzyme levels in response to changes in physiological state of the animal involved new enzyme synthesis.

To determine whether changes in the rate of degradation of acetyl CoA carboxylase contribute to the alterations in hepatic enzyme activity Nakaniski and Numa (90) labeled the enzyme with [^{3}H] leucine and determined the decay of radioactivity in the carboxylase, isolated by immunoprecipitation. They found that the half-life of the enzyme did not vary significantly in normal, fat-free-refed, or diabetic rats, but that there was marked acceleration of the rate of degradation in the liver of fasted animals. Thus, the change in carboxylase content of animals in a steady state (i.e. refed or diabetic animals) is due to a rise or fall in the rate of enzyme synthesis, whereas the change in enzyme content of animals, which are not in a steady state (i.e. fasted animals) is due both to diminished enzyme synthesis and to accelerated enzyme degradation.

Genetic and Hormonal Factors

Correlations of enzyme activities similar to those observed in genetic states such as hereditary obesity have been observed. Acetyl CoA carboxylase, citrate cleavage enzyme, and fatty acid synthetase were found to be elevated in obese mice (94). In addition coordinated changes in lipogenic enzyme activity have been noted in certain hormonal disturbances such as diabetes mellitus and in response to hormone treatment. Nepokroeff et al. (95) have demonstrated that insulin is obligatory for the coordinated induction of the following hepatic lipogenic enzymes during the refeeding of a fat-free diet to previously fasted diabetic rats: acetyl CoA carboxylase, citrate cleavage enzyme, malic enzyme, 6- phosphogluconate dehydrogenase and glucose - 6 - phosphate dehydrogenase. However, under these same conditions there was found a lack of response of cytosol isocitrate dehydrogenase to insulin, which may indicate that this enzyme is not coordinately regulated with the lipogenic enzymes and that it is not as important as other NADPH-generating enzymes in providing reducing equivalents for the enhanced lipogenesis that occurs during fat-free diet refeeding.

Nepokroeff et al. (95) also found a distinct inhibition of each of the lipogenic enzymes by glucagon in normal animals during fat-free diet refeeding. These findings support the concept that the relative concentrations of insulin and glucagon are important in the coordinate control of the lipogenic enzymes. Further support of this concept is provided by the findings of Volpe and Marasa (96). They observed that the administration of glucagon during the refeeding of starved rats

resulted in a marked inhibition of the induction of acetyl CoA carboxylase, fatty acid synthetase and the rate of incorporation of tritium from tritiated water into fatty acids in liver. However, they found no change in these parameters in adipose tissue.

Volpe and Marasa (96) also found a marked reduction in the activities of acetyl CoA carboxylase and fatty acid synthetase in adipose tissue with no change in liver following the administration of glucocorticoids. In addition an increase in activities of these two enzymes in adipose tissue but with no noted change in liver was observed following adrenalectomy.

It was observed that thyroxine injection into rats resulted in an increase in liver citrate cleavage enzyme activity and hypophysectomy resulted in a decrease in liver, adipose, and adrenal gland citrate cleavage enzyme activity, which was restored upon thyroxine injection (67, 97). Thyroxine was also found to induce hepatic acetyl CoA carboxylase and malic enzyme activities (98).

Developmental Factors

Acetyl CoA carboxylase was reported to rise dramatically in rat and mouse liver at the time of weaning, in avian liver after hatching, in rat and rabbit mammary gland during mid-to late pregnancy and again early in lactation (85). Rat liver malic enzyme has been found to increase rapidly after weaning, reaching a maximum after a few days and then gradually decreasing to reach adult levels (99). Since in these studies the animals changed from a high-fat to a relatively low-fat diet at the time of weaning and hatching, the rise

in enzyme levels may be related to the level of dietary fat (85, 86). In support of this explanation are the findings that glucose injection into embryonic eggs resulted in an increase in the level of hepatic acetyl Co carboxylase and that weaning mice prematurely to a fat-free diet resulted in an increase in the level of hepatic acetyl CoA carboxylase and citrate cleavage enzyme, respectively (100, 101).

CHAPTER III

EXPERIMENTAL PROCEDURE

I. General Plan

The purpose of this study was to investigate the effect of various ratios and sources of inorganic and neutral sulfur on lipid metabolism in the rat liver utilizing the enzyme systems of acetyl CoA carboxylase, citrate cleavage enzyme, and malic enzyme. The liver was chosen as the site for investigation since these three enzymes have been studied extensively in this tissue.

Randomly selected adult male rats of a Wistar, Sprague-Dawly cross strain served as tissue donors. The animals were housed in galvanized steel cages according to diet with no more than five animals per cage. All rats were killed between 8:00 and 9:00 in the morning to avoid diurnal variation. The rats were stunned by a blow to the head, decapitated, and their livers were removed to a cold buffer solution for subsequent removal of nuclei and mitochondria. Enzyme assays of the resulting cytoplasmic preparations were made. Although the detailed procedures for determining the activity of acetyl CoA carboxylase, citrate cleavage enzyme and malic enzyme as well as other determinations are discussed in the methods section of this chapter, the general principles of the enzyme determinations will be discussed in this section.

Acetyl CoA carboxylase activity was assayed according to the

method described by Inoue and Lowenstein (102) which is based on the principle that ^{14}C- bicarbonate is converted into the carboxyl group of malonyl CoA which results in the conversion of an acid-volatile compound into an acid stable compound. Using this method the enzyme was first activated by incubation at 37° with citrate and magnesium ions for 15 minutes followed by the addition of substrate, ^{14}C- bicarbonate, and ATP, and a continuation of incubation for 10 minutes. The reaction was stopped by the addition of perchloric acid. The reaction mixture was then taken to dryness which allowed the unreacted ^{14}C- bicarbonate to escape as $^{14}CO_2$ and the fixed ^{14}C in malonyl CoA to remain. The radioactivity of the residue was determined by liquid scintillation spectrophotometry and was taken as a measure of the activity of the enzyme.

Citrate cleavage enzyme activity was assessed according to the method described by Cottam and Srere (64) which monitors the formation of oxalacetate by its reaction with NADH in the presence of malate dehydrogenase. The rate of disappearance of NADH was observed with a Beckman DU[12] spectrophotometer at a wavelength of 340nm. Thus the change in absorption at 340nm was taken as a measure of the activity of the enzyme.

Malic enzyme activity was assessed according to the method outlined by Ochoa (103) which is based on the fact that the rate of reduction of NADP, in the presence of the enzyme, and an excess

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[12]Beckman DU Spectrophotometry, Palminalto, California as modified by Update Instrument Company, Madison, Wisconsin.

of L-malate and Mn^{++}, is proportional to the enzyme concentration. The rate of formation of NADPH was observed in the Beckman DU spectrophotometer at a wavelength of 340nm.

II. Methods

Diets

All rats were fed Rat Chow[13] and tap water ad libitum from weaning to adult (200-250 grams). At this time, they were fed a semi-purified diet consisting of the basal diet described in table 1 plus one of the variations shown in table 2 and distilled water ad libitum. The calculated levels of inorganic sulfate, organic, and total sulfur as sulfate of the semi-purified diets is given in table 3. The rats were fed the semi-purified diet for 17 days since metabolic alterations which were due to the level of inorganic sulfate in the diet have been detected within a period of 14 days when rats were fed the diets shown in tables 1 and 2 (6). The diets shown in table 1 are a variation of the diets of Caputto et al. (104). The salt mixture is that of Hubbell et al. (105) as modified by Pendergrass.[14]

Preparation of Liver Homongenate

Following decapitation of the rats and removal of the liver to a cold solution of either 0.1M Tris-HCl [tris (hydroxymethyl

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

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

Table 1

Invariable Composition of Semi-Purified Diets

Component	g/100g diet
Casein[1]	15.00
Cornstarch	30.00
Sucrose	30.00
Vegetable oil[2]	2.00
Vegetable shortening[3]	10.00
Vitamin mixture[4]	2.00
Basic salt mixture[5]	1.34
Variable components	see Table 2

[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 9.090, vitamin B_{12} 0.00135, α-tocopherol 5.0, vitamin A 9 x 10^5 units, vitamin D 1 x 10^5 units, and sufficient glucose to make 1 kg.

[5]1.34 g 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 $MnCl_2 \cdot 6H_2O$.

Table 2

Variations of the Basal Diet

Diet	Dietary Components					
	Inorganic $SO_4^=$	$CaSO_4 \cdot 2H_2O$	$CaCO_3$	DL Methionine[1]	Cysteine[1] free base	Non-Nutritive bulk[2]
	%	g/100g	g/100g	g/100g	g/100g	g/100g
A	0.0002	0.0	1.34	0.66	0.0	7.66
B	0.0002	0.0	1.34	0.62	0.0	7.70
C	0.0002	0.0	1.34	0.0	0.53	7.79
D	0.0002	0.0	1.34	0.0	0.505	7.82
E	0.02	0.04	1.32	0.62	0.0	7.68
F	0.02	0.04	1.32	0.0	0.505	7.80
G	0.42	0.75	0.92	0.0	0.0	8.00
H	0.42	0.75	0.92	0.62	0.0	7.38
I	0.42	0.75	0.92	0.0	0.505	7.50

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

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

Table 3

Calculated Levels of Inorganic, Organic, and Total Sulfur as Sulfate of the Semi-purified Diets

Diets	Inorganic $SO_4^=$	Organic Sulfur as $SO_4^=$	Total Sulfur as $SO_4^=$
	% of diet	% of diet	% of diet
A	0.0002	0.67	0.67
B	0.0002	0.65	0.65
C	0.0002	0.67	0.67
D	0.0002	0.65	0.65
E	0.02	0.65	0.67
F	0.02	0.65	0.67
G	0.42	0.25	0.67
H	0.42	0.65	1.07
I	0.42	0.65	1.07

aminomethane)], buffer, pH 7.5 or 0.1M imidazole - HCl, pH 7.5, the net weight of each liver was determined using a top-loading balance and recorded to the nearest gram. The livers and subsequent fractions were kept in an ice bath throughout the procedure. Ten percent liver homogenates were prepared with cold 0.1M Tris-HCl [tris (hydroxymethyl aminomethane)] buffer pH 7.5 or 0.1M imidazole - HCl pH 7.5. All homogenizations were carried out using a motor driven homogenizer[15] with a Teflon pestle. The homogenate was centrifuged at 12,000 x g in a refrigerated centrifuge[16] for 25 minutes at 4° to sediment nuclei and mitochondria. The supernatant fluid was decanted and saved for enzyme assays. The assay of acetyl CoA carboxylase, citrate cleavage enzyme, and malic enzyme activity of this cytoplasmic preparation was immediately made.

Enzyme Assays

Determination of liver acetyl CoA carboxylase activity from rats fed various ratios and sources of inorganic and netural sulfur. Acetyl CoA carboxylase activity was assessed according to the method described by Inoue and Lowenstein (102). The preincubation medium contained in a final volume of 2.2 ml the constituents listed in table 4. The incubation medium contained in a final volume of 2.0 ml the components listed in table 5. All assays were run in duplicate, and duplicate blanks, without acetyl CoA, were run with each

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[15]Thomas Size C Homogenizer, Thomas Co., Philadelphia, Pa.

[16]Lourdes Betafuze Model A, Lourdes Instrument Corporation, Brooklyn, N.Y.

Table 4

Components of the Preincubation Mixture
Used to Determine Acetyl CoA Carboxylase Activity

Component	Concentration[1]
	u moles/incubation
Imidazole - HCl, pH 7.5[2]	330
Potassium citrate	165
$MgCl_2 \cdot 6H_2O$	132
2-Mercaptoethanol[2]	1.54

[1]Concentration of components in u moles in 2.2 ml final volume.

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

Table 5

Components of the Incubation Mixture Used to Determine Acetyl CoA Carboxylase Activity

Component	Concentration[1]
	u moles/incubation
Acetyl CoA[2]	7
Na_2ATP[2]	80
$NaH^{14}CO_3$[3]	75

[1]Concentration of components in u moles in 2.0 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 1.6 x 10^5 cpm/uM bicarbonate.

series of assays. To small tubes (12 x 100 mm) the following was added: 0.2 ml preincubation medium, 0.03 ml of 1% bovine serum albumin, and water to a volume of 0.39 ml. The tubes were placed in an Eberbach water-bath shaker at 37°. The preincubation was initiated with 10 ul of a 10% cytoplasmic preparation of the liver homogenate prepared as described previously and allowed to proceed for 15 minutes. The assay was then initiated with 0.1 ml of incubation medium. After 10 minutes the reaction was terminated with 0.05 ml of 4 N $HClO_4$. The tubes were placed in an ice bath and excess ClO_4^- was precipitated by adding 0.1 ml of 2 M KCl. The samples were removed from the ice bath, covered, and were allowed to sit at least 24 hours at room temperature. Either 50 ul or 100 ul of the resultant supernatant fluid from each sample was spotted on 1 x 4.5 cm strips of Whatman #3 filter paper. All of the strips of paper were allowed to dry for an equal period of time (for at least 30 minutes). Each strip was placed in a scintillation vial containing a cocktail of 12 g PPO^- (2, 5, diphenyl oxazol) per liter of toluene. The samples were then counted in a Picker Nuclear Liquimat 220 Liquid Scintillation Spectrometer. Quenching by the paper strips was negligible.

The enzyme activity of acetyl CoA carboxylase was expressed as u moles of CO_2 fixed per mgN per minute as calculated from the specific activity of the $H^{14}CO_3^-$ as shown in the following formula.

$$uM/mgN = \frac{CPM/mgN}{CPM/uM \text{ of } H^{14}CO_3^-}$$

Determinations of liver citrate cleavage enzyme activity from rats fed various ratios and sources of inorganic and netural sulfur. The activity of citrate cleavage enzyme was determined according to

the method described by Cottam and Srere (64). Each reaction tube contained in a final volume of 2.9 ml the constituents listed in table 6 and 0.1 ml of a 10% cytoplasmic preparation of the liver homogenate prepared as described previously. All assays were run in duplicate. Reactions were initiated with coenzyme A and malate dehydrogenase. Absorbance readings were made at room temperature (20-25°) and at a wavelength of 340nm, against a blank containing all components except coenzyme A and malate dehydrogenase, at 10 minute invervals for 20 minutes. The change in absorbance reading over the first 10 minute period was used to determine the average change in absorbance per minute.

Enzyme activity was expressed as units of activity per mgN per minute. One enzyme unit of activity was defined as the change in absorbance of 0.001 per minute at 340nm per mg of nitrogen (106).

Determination of liver malic enzyme activity from rats fed various ratios and sources of inorganic and neutral sulfur. The activity of malic enzyme was determined by the method described by Ochoa (103). Each reaction tube contained in a final volume of 3.0 ml the constituents in table 7 and 0.1 ml of a 10% cytoplasmic preparation of the liver homogenate prepared as described previously. All assays were preformed in duplicate. The reaction was initiated by the addition of 0.1 ml of enzyme preparation. Absorbance readings were made at room temperature (20-25°) and at a wavelength of 340 nm, against a blank containing all components except NADP, at 10 minute intervals for 20 minutes. The change in absorbance reading over the first 10 minute period was used to determine the average change in

Table 6

Components of the Reaction Mixture Used to Determine Citrate Cleavage Enzyme Activity

Component	Concentration[1]
	u moles/incubation
Tris - HCl, pH 8.7[2]	100
Potassium citrate	20
$MgCl_2$	10
Dithiothreitol[2]	10
Coenzyme A[2]	0.33
NADH[2]	0.14
Na_2ATP[2]	5
Malate Dehydrogenase[3] (0.5 IU)	

[1]Concentration of components is u moles in 3.0 ml final volume.

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

[3]0.5 international units, 0.6 lamda/incubation.

Table 7

Components of the Reaction Mixture Used to Determine Malic Enzyme Activity

Component	Concentration[1]
	u moles/incubation
glycylglycine, pH 7.4[2]	75
$MnCl_2$	3
NADP[2]	0.135
L-malate, pH 7.4[2]	1.5

[1]Concentration of components is u moles in 2.9 ml final volume.

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

absorbance per minute.

Enzyme activity was expressed as units of activity per mgN per minute. One enzyme unit was defined as the amount of enzyme causing a change in absorbance of 0.01 per minute under the conditions of the assay.

Determination of the Nitrogen Content of the Enzyme Solutions

The nitrogen or protein concentrations of the cytoplasmic enzyme preparation was determined using the micro-Kjeldahl method as standardized by Willets and Ogg (107) or the Folin-Ciocalteau method for protein determination as described by Lowry et al. (108), respectively.

Micro-Kjeldahl method - One ml of the cytoplasmic enzyme preparation was pipetted into a 30 ml micro-Kjeldahl flask containing 1.25 to 1.36g 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 digested until clear (approximately 15 hours) on a micro-Kjeldahl heating apparatus. After the flasks were cooled, and upon the addition of 5 ml of distilled water to dissolve the solids, the contents were transferred to a standard micro steam distillation apparatus. Completeness of transfer of the digest was tested by the addition of a drop of methyl orange indicator to the final rinse. A 125-ml Erlenmeyer receiver flask containing 5 ml of a 4% boric acid solution and 4 drops of methyl red - bromocresol green indicator (0.83% bromocresol green and 0.17g methyl red diluted to 500 ml with 95% ethanol) was placed under the condenser with the tip extending below the surface. Following the addition of 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) to the apparatus, the

distillation process was begun and proceeded until approximately 50 ml of distillate had been collected. The nitrogen content of the distillate was determined by titrations of the distillate with 0.010 N HCl (3.6g constant boiling HCl diluted to 2000 ml with distilled water). Duplicates were run for each sample as well as a blank which contained all reagents except the enzyme preparation. Milligrams of nitrogen were calculated by the following formula: (ml HCl) x (N of acid) x mEq weight of nitrogen) = mg nitrogen.

Folin-Ciocalteau method One ml samples were prepared so that they contained 30 - 200 ug of protein in 1 ml of 0.5N NaOH. This was done by combining 1 ml of the cytoplasmic preparation with 1 ml of 1.0N NaOH and then taking 0.2 ml of this mixture and bringing it to a volume of 6 ml with 0.5N NaOH. One ml aliquots were pipetted into test tubes. All assays were preformed in duplicate.

Five ml of reagent A (prepared by adding 1 ml of 2.7% sodium potassium tartrate $.4H_2O$ and then 1 ml of 1% Cu SO_4 . $5H_2$ to 100 ml of 2% Na_2CO_3) were mixed with each sample and allowed to stand at room temperature for at least 10 minutes. Reagent B was prepared by diluting commercial Folin-Ciocalteau reagent with enough distilled water so it contained 1N acid. Five-tenths ml of reagent B was quickly added and mixed within 1 second. After at least 30 minutes, the absorbance was determined at 750 nm using a Beckman B spectrophotometer. The protein concentration (mg protein/ml sample) of the sample was calculated by comparing the absorbance of the sample to that of the protein standard as shown below:

$$\frac{abs_{sample}}{abs_{std.}} \text{ X conc.}_{std.} \text{ x 60 = mg protein/ml}$$

Then the calculated value was divided by 6.25 in order to determine mg of nitrogen per ml of sample.

The protein standard was prepared by weighing 0.02g of bovine albumin and diluting to a volume of 100 ml with 0.5N NaOH giving a concentration of 200 ug of protein per ml. One ml of diluted standard was treated in the same manner as the samples.

Statistics

The data were analyzed by using either the t-test for paired comparisons, Duncan's new multiple range for equal replications, or Duncan's new multiple range for unequal replications as described by Steele and Torrie (109). All computations were made using the Olivette-Underwood Programmer 101.

CHAPTER IV

Results

The purpose of this investigation was to study the effect of feeding diets containing varying ratios and sources of neutral and inorganic sulfur on the activities of three enzymes involved in lipogenesis. Acetyl CoA carboxylase, citrate cleavage enzyme, and malic enzyme were chosen.

I. The effect of varying the level of dietary inorganic sulfate and neutral sulfur on acetyl CoA carboxylase activity

An investigation of the effect of feeding diets containing varying ratios of neutral to inorganic sulfur on the activity of acetyl CoA carboxylase, the rate limiting enzyme of fatty acid synthesis, was conducted. These diets contained inorganic sulfate and methionine supplemented in inverse amounts thus producing inorganic sulfate levels of 0.0002% (diet A), 0.02% (diet E), and 0.42% (diet G) while maintaining the total sulfur as sulfate level constant. The results of an evaluation of the activity of acetyl CoA carboxylase from the livers of rats fed diets A, E, and G are shown in table 8. These data show that there is a decrease in the activity of the enzyme when inorganic sulfate is fed at levels above and below the established optimum of 0.02%.

Since an effect on the activity of acetyl CoA carboxylase was observed when rats were fed diets in which calcium sulfate and methionine were varied inversely to maintain the total sulfur as

Table 8

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Methionine Upon the Activity of Rat Liver Acetyl CoA Carboxylase When the Total Sulfur as Sulfate Level Was Kept Constant

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Acetyl CoA Carboxylase Activity	
	%	%	n moles/mgN[1]	
A	0.0002	0.67	28 ± 4^{ab}[2]	(8)[3]
E	0.02	0.65	36 ± 8^{b}	(8)
G	0.42	0.25	22 ± 6^{a}	(7)

[1] Values are means $\pm$ SEM.

[2] All means not followed by the same superscript are significantly different ($P < 0.01$) by the method of Duncan's new multiple range for unequal replications (109).

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

sulfate level constant, an investigation of the effect of feeding diets containing constant amounts of methionine and the levels of inorganic sulfate previously used was made. The data presented in table 9 show the activity of acetyl CoA carboxylase from the livers of rats fed diets B, E, and H. An examination of these data indicates that when inorganic sulfate is fed at levels above as well as below the optimum that there is an increase in the activity of the enzyme. A comparison of the data presented in table 8 with those in table 9 shows that the activity of acetyl CoA carboxylase is decreased when rats are fed a high level of inorganic sulfate (0.42%) and an inadequate level of neutral sulfur (diet G) as opposed to a high sulfate level and an optimum level of neutral sulfur (diet H).

In order to investigate the effect of varying the source of dietary neutral sulfur on the activity of acetyl CoA carboxylase, rats were fed diets supplemented with cysteine and the activity of the liver enzyme was evaluated. The diets which were fed contained inorganic sulfate and cysteine supplemented in inverse amounts yielding inorganic sulfate levels of 0.0002% (diet C), 0.02% (diet F) and 0.42% (diet G) while maintaining the total sulfur as sulfate level constant. The data presented in table 10 show that when dietary inorganic sulfate is fed at levels above and below the optimum that there is a decrease in the activity of the enzyme. A comparison of the data in table 8 with those in table 10 indicate that the same trend in the effect of dietary treatment on the activity of acetyl CoA carboxylase occurred even though the source of neutral sulfur was different.

In order to evaluate the effect on the activity of acetyl CoA

Table 9

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Constant Levels of Dietary Methionine Upon the Activity of Rat Liver Acetyl CoA Carboxylase

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Acetyl CoA Carboxylase Activity	
	%	%	n moles/mgN[1]	
B	0.0002	0.65	20 ± 3^{a2}	(7)[3]
E	0.02	0.65	18 ± 3^{a}	(7)
H	0.42	0.65	28 ± 6^{a}	(7)

[1]Values are means $\pm$ SEM.

[2]All means not followed by the same superscript are significantly different ($P < 0.05$) by the t-test for paired comparisons (109).

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

Table 10

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Cysteine Upon the Activity of Rat Liver Acetyl CoA Carboxylase When the Total Sulfur as Sulfate Level Was Kept Constant

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Acetyl CoA Carboxylase Activity	
	%	%	n moles/mgN[1]	
C	0.0002	0.67	24 ± 8^{a}[2]	(7)[3]
F	0.02	0.65	28 ± 8^{a}	(7)
G	0.42	0.25	24 ± 8^{a}	(7)

[1] Values are means $\pm$ SEM.

[2] All means not followed by the same superscript are significantly different ($P < 0.05$) by the t-test for paired comparisons (109).

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

carboxylase of varying the level of dietary inorganic sulfate with cysteine supplementation held constant, diets containing equal amounts of cysteine and levels of inorganic sulfate previously used [0.0002% (diet D), 0.02 (diet F),and 0.42 (Diet I)] were fed. The data presented in table 11 indicate that inorganic sulfate when fed at levels below and above the optimum of 0.02, causes a decrease in activity of the enzyme. A reverse effect on the activity of the enzyme is shown when the source of neutral sulfur is changed from methionine (table 9) to cysteine (table 11).

II. The effect of varying the level of dietary inorganic sulfate and neutral sulfur on citrate cleavage enzyme activity

Citrate cleavage enzyme is involved in the generation of acetyl CoA, a precursor of fatty acid synthesis. Therefore, the effect on the activity of citrate cleavage enzyme of feeding the diets previously discussed in connection with acetyl CoA carboxylase was investigated. When rats were fed diets containing constant levels of total sulfur as sulfate and varying levels of inorganic sulfate and methionine, the activity of citrate cleavage enzyme was decreased at the 0.0002% and 0.42% levels as compared to the optimum level (table 12). A similar effect was observed when constant amounts of methionine and the varying levels of inorganic sulfate were fed (table 13). A comparison of the data presented in table 12 with those in table 13 indicates that the activity of citrate cleavage enzyme is higher when rats are fed a high inorganic sulfate level (0.42%) along with a low level of methionine (diet G) versus

Table 11

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Constant Levels of Dietary Cysteine Upon the Activity of Rat Liver Acetyl CoA Carboxylase

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Acetyl CoA Carboxylase	
	%	%	n moles/mgN[1]	
D	0.0002	0.65	16 ± 3^{ab}[2]	(9)[3]
F	0.02	0.65	23 ± 4^{b}	(9)
I	0.42	0.65	16 ± 3^{a}	(9)

[1] Values are means $\pm$ SEM.

[2] All means not followed by the same superscript are significantly different ($P < 0.01$) by the t-test for paired comparisons (109).

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

Table 12

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Methionine Upon the Activity of Rat Liver Citrate Cleavage Enzyme When the Total Sulfur as Sulfate Level Was Kept Constant

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Citrate Cleavage Enzyme	
	%	%	units/mg N/min[1]	
A	0.0002	0.67	290 ± 38[a2]	(8)[3]
E	0.02	0.65	304 ± 37[a]	(8)
G	0.42	0.25	295 ± 50[a]	(7)

[1]Values are means ± SEM.

[2]All means not followed by the same superscript are significantly different ($P < 0.05$) by the t-test for paired comparisons (109).

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

Table 13

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Constant Levels of Dietary Methionine Upon the Activity of Rat Liver Citrate Cleavage Enzyme

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Citrage Cleavage Enzyme	
	%	%	units/mgN/min[1]	
B	0.0002	0.65	215 ± 38[ab2]	(7)[3]
E	0.02	0.65	225 ± 28[b]	(7)
H	0.42	0.65	178 ± 29[a]	(7)

[1]Values are means ± SEM.

[2]All means not followed by the same superscript are significantly different ($P < 0.05$) using the t-test for paried comparisons (109).

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

a high sulfate level with optimum methionine supplementation (diet H).

The activity of citrate cleavage enzyme was found to increase as the level of dietary inorganic sulfate increased when rats were fed diets containing calcium sulfate and cysteine which were varied inversely in order to keep the total level of sulfur as sulfate constant (table 14). However, when rats were fed diets in which cysteine was kept constant while the level of dietary inorganic sulfate was increased, the activity of citrate cleavage enzyme was decreased at levels above as well as below the optimum of 0.02% (table 15).

III. The effect of varying the level of dietary inorganic sulfate and neutral sulfur on malic enzyme activity

Malic enzyme is involved in the generation of a reducing equivalent, NADPH, for fatty acid synthesis. Therefore, determinations of the activity of malic enzyme from the same rat livers which served as the enzyme source for acetyl CoA carboxylase and citrate cleavage enzyme assays were made. The data in table 16 show that the activity of malic enzyme from the livers of rats fed inorganic sulfate levels of 0.0002% (diet A) and 0.42% (diet G) was increased in comparison with that from the livers of rats fed 0.02% sulfate (diet E). However, when methionine supplementation was kept constant malic enzyme activity was increased over the optimum only at the 0.0002% sulfate level (table 17). Furthermore, a comparison of the data in table 16 with those in table 17 shows that the activity of

Table 14

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Cysteine Upon the Activity of Rat Liver Citrate Cleavage Enzyme When the Total Sulfur as Sulfate Level Was Kept Constant

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Citrate Cleavage Enzyme	
	%	%	units/mgN/min[1]	
C	0.0002	0.67	199 ± 37[ab2]	(6)[3]
F	0.02	0.65	211 ± 26[b]	(6)
G	0.42	0.25	317 ± 28[a]	(6)

[1]Values are means ± SEM.

[2]All means not followed by the same superscript are significantly different ($P < 0.05$) using the t-test for paired comparisons (109).

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

Table 15

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Constant Levels of Dietary Cysteine Upon the Activity of Rat Liver Citrate Cleavage Enzyme

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Citrate Cleavage Enzyme	
	%	%	units/mgN/min[1]	
D	0.0002	0.65	225 ± 19[a2]	(7)[3]
F	0.02	0.65	334 ± 34[b]	(7)
I	0.42	0.65	248 ± 46[ab]	(7)

[1]Values are means ± SEM.

[2]All means not followed by the same superscript are significantly different ($p < 0.01$) using the t-test for paired comparisons (109).

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

Table 16

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Methionine Upon the Activity of Rat Liver Malic Enzyme When the Total Sulfur as Sulfate Level Was Kept Constant

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Malic Enzyme Activity	
	%	%	units/mgN/min[1]	
A	0.0002	0.67	10 ± 4^{a}[2]	(7)[3]
E	0.02	0.65	6 ± 1^{a}	(7)
G	0.42	0.25	9 ± 1^{a}	(7)

[1]Values are means $\pm$ SEM.

[2]All means not followed by the same superscript are significantly different ($P < 0.05$) by the t-test for paired comparisons (109).

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

Table 17

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Constant Levels of Dietary Methionine Upon the Activity of Rat Liver Malic Enzyme

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Malic Enzyme Activity	
	%	%	unit/mgN/min[1]	
B	0.0002	0.65	7 ± 1^{a}[2]	(6)[3]
E	0.02	0.65	5 ± 1^{b}	(6)
H	0.42	0.65	5 ± 1^{ab}	(6)

[1]Values are means $\pm$ SEM.

[2]All means not followed by the same superscript are significantly different ($P < 0.01$) using the t-test for paired comparisons (109).

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

malic enzyme is higher when rats are fed a high inorganic sulfate level along with a low level of methionine versus a high sulfate level with optimum supplementation of methionine. Malic enzyme activity from the livers of rats fed varying amounts of cysteine and inorganic sulfate and constant levels of total sulfur as sulfate was increased at both low and high inorganic sulfate levels compared to the 0.02% levels (table 18). A comparison of the effect on liver malic enzyme activity when rats were fed constant levels of methionine (table 17) versus constant levels of cysteine (table 19) shows an activation in the case of the former and an inhibition in the case of the latter at levels of inorganic sulfate above and below the optimum. In addition, a reverse effect on the activity of malic enzyme occurred when cysteine levels were held constant as inorganic sulfate was varied (table 19). As in the case of methionine supplementation, the addition of an optimum level of cysteine versus no cysteine addition to a diet containing a high level of inorganic sulfate resulted in a decreased malic enzyme activity in those rats fed diets with cysteine.

Table 18

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Cysteine Upon the Activity of Rat Liver Malic Enzyme When the Total Sulfur as Sulfate Level was Kept Constant

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Malic Enzyme Activity
	%	%	units/mgN/min[1]
C	0.0002	0.67	8 ± 3^{ab}[2] (7)[3]
F	0.02	0.65	5 ± 1^{b} (7)
G	0.42	0.25	9 ± 1^{a} (7)

[1] Values are means $\pm$ SEM.

[2] All means not followed by the same superscript are significantly different ($P < 0.01$) using the t-test for paired comparisons (109).

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

Table 19

Effect of Feeding Different Levels of Dietary Inorganic Sulfate and Constant Levels of Dietary Cysteine Upon the Activity of Rat Liver Malic Enzyme

Diet	Inorganic Sulfate	Neutral Sulfur as Sulfate	Malic Enzyme Activity	
	%	%	units/mgN/min[1]	
D	0.0002	0.65	5 ± 2^{a}[2]	(7)[3]
F	0.02	0.65	7 ± 2^{a}	(7)
I	0.42	0.65	5 ± 1^{a}	(7)

[1]Values are means $\pm$ SEM.

[2]All means not followed by the same superscript are significantly different ($P < 0.05$) by the t-test for paired comparisons (109).

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

CHAPTER V

DISCUSSION

Previous investigations in this laboratory have documented that metabolic alterations occur in rats fed diets containing inorganic sulfate at levels above or below the established optimal level of 0.02%. In addition, dietary inorganic sulfate has been shown to influence carbohydrate metabolism. The present investigation has extended these studies to include lipogenesis.

Data have been presented which show that inorganic sulfate, when fed at levels above or below the established dietary optimum of 0.02%, caused a decrease in the activity of acetyl CoA carboxylase (table 8, page 64) and citrate cleavage enzyme (table 12, page 70). However, under the same dietary conditions the activity of malic enzyme (table 16, page 75) was increased.

An increase in the activity of phosphoenol pyruvate carboxykinase and pyruvate carboxylase, enzymes of gluconeogenesis, and an increase in the levels of blood glucose and liver glycogen have been found to occur when inorganic sulfate comprises 0.42% of the diet. Therefore, the decreased activity of acetyl CoA carboxylase and citrate cleavage enzyme under these same conditions is reasonable since these two enzymes may act as controlling sites for fatty acid synthesis and since gluconeogenesis and fatty acid synthesis would not occur under the same physiological conditions.

However, the activity of malic enzyme, another supporting enzyme of fatty acid synthesis was not affected in a manner similar to the other two enzymes. As previously discussed Stark et al.(78) found that under conditions of protein restriction there was a positive correlation between the activities of malic enzyme and glutathione reductase but no detectable correlation between malic enzyme activity and lipogenesis suggesting that malic enzyme may be involved in furnishing reducing equivalents required for the formation of reduced glutathione rather than for fatty acid synthesis. Thus malic enzyme may have other physiological functions besides that of its role in lipogenesis.

If malic enzyme is not involved in the generation of NADPH under the conditions of this investigation then the question arises as to the source of the reducing equivalents for fatty acid biosynthesis. Other enzymes involved in the synthesis of the reducing agent, NADPH, are those of the hexose monophosphate shunt (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) and cytoplasmic isocitrate dehydrogenase. Since cytoplasmic isocitrate dehydrogenase has not been found to fluctuate with fatty acid synthesis or other lipogenic enzymes under various metabolic states, it is doubtful that this enzyme is the source of the reducing equivalents for lipogenesis. However, the activity levels of the dehydrogenase enzymes of the hexose monophosphate shunt have been found to undergo coordinate changes with fatty acid synthesis and other lipogenic enzyme levels.

"Active sulfate" (3'-phosphoadenosine- 5'-phosphosulfate,

PAPS) serves as the sulfate donor in several reactions in the mammalian liver. The sulfate activation mechanism has been found to require three ATP equivalents. Ribose-5-phosphate is a product of the hexose monophosphate shunt and is the precursor of 5-phosphoribosyl-1-pyrophosphate (PRPP) which is required for *de novo* synthesis of purine and pyrimidine nucleotides. Thus sulfate activation would increase the need for *de novo* synthesis of ATP which would increase the need for ribose-5-PO_4 and thus in turn increase the activity levels of enzymes of the hexose monophosphate shunt and the supply of NADPH.

Data have been presented (table 9 page 66) which show that inorganic sulfate, when fed with adequate amounts of methionine at levels above or below the optimum cause an increase in the activity of acetyl CoA carboxylase. White[16] noted similar results for propionyl CoA carboxylase activity and proposed that the mechanism of the effect of very low levels of dietary inorganic sulfate (0.0002%) upon this enzyme activity was related to an increased availability of a cytoplasmic factor necessary for enzyme synthesis. This cytoplasmic factor was suggested to influence the rate of binding of biotin to the epsilon amino group of the lysine residue of the enzyme protein. Since biotin is required in the synthesis of the acetyl CoA carboxylase holoenzyme (10) and similar effects of diets on propionyl CoA carboxylase activity as that for acetyl CoA

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[16]See footnote 3 page 4.

carboxylase activity was observed, White's explanation of the effect of very low levels of dietary inorganic sulfate on the activity of propionyl CoA carboxylase may also apply to acetyl CoA carboxylase.

White also investigated the mechanism of the increase in propionyl CoA carboxylase activity observed when inorganic sulfate was fed at levels above the dietary optimal and found that inorganic sulfate allosterically activated the enzyme. The increased activity of acetyl CoA carboxylase found to occur when inorganic sulfate comprised 0.42% of the diet may be accomplished allosterically. Citrate has been shown to allosterically activate acetyl CoA carboxylase by shifting the protomer-polymer equilibrium toward the catalytically active state by inducing a conformational change in the carboxylase protomer which favors polymerization (10). Gregolin et al. (22) studied *in vitro* factors affecting the interconversion between the protomeric and polymeric forms of avian enzyme and found that sulfate as well as certain other anions promoted aggregation of the protomer. Thus, activation of acetyl CoA carboxylase by high levels of dietary inorganic sulfate may be accomplished in a manner similar to that shown for propionyl CoA carboxylase. High levels of sulfate may induce a conformational change in the enzyme protein in a manner similar to that induced by citrate, that is the carboxybiotin prosthetic group may be brought into closer proximity to the substrate binding sites or sulfate may enhance the affinity of a binding site for the ureido ring of biotin and thus increase the rate of biotin binding.

An effect similar to that observed for acetyl CoA carboxylase, when rats were fed diets containing varying levels of inorganic sulfate

supplemented with at least adequate amounts of methionine, was observed for malic enzyme only at the 0.0002% $SO_4^=$ level. Thus it appears that malic enzyme activity could supply the reducing equivalents for fatty acid biosynthesis indicated by the activity of acetyl CoA carboxylase in the livers of rats fed diets low in sulfate but supplemented with methionine. However, since there was no difference observed in the activity of malic enzyme at the 0.42% $SO_4^=$ level and the 0.02% level, it appears that malic enzyme could not supply the reducing equivalents for the biosynthesis of fatty acids indicated by the acetyl CoA carboxylase activity in the livers of rats fed the high sulfate level supplemented with methionine.

When rats were fed diets containing 0.42% $SO_4^=$ level supplemented with at least adequate amounts of methionine the activity of acetyl CoA carboxylase was increased by 56% over the optimum level whereas citrate cleavage enzyme was decreased 21%. Thus the question arises as to the source of acetyl CoA for fatty synthesis under these dietary conditions. In the catabolic pathway of methionine, a cleavage of cysthathionine with the formation of cysteine and a 4 carbon fragment occurs. The exact fate of this carbon fragment is not known. It may be that when large amounts of sulfur are provided in the diet, that methionine may be metabolized to acetyl CoA. Another possible explanation is that in the presence of such a large amount of sulfur, citrate cleavage enzyme inhibition results in a lack of substrate for fatty acid synthesis and even though acetyl CoA carboxylase activity is increased, fatty acid synthesis may not occur at a rate equivalent to enzyme activity. This explanation is supported by the fact that both acetyl CoA carboxylase

and citrate cleavage enzyme are inhibited when rats are fed diets supplemented with cysteine and containing inorganic sulfate above or below the optimum.

Previous investigations have shown that cysteine supplementation is not metabolically equivalent to methionine supplementation. Cysteine was substituted for methionine in the diets previously discussed to assess the effect on enzyme activity of different sources of neutral sulfur. Cysteine supplementation resulted in a decrease in the activities of acetyl CoA carboxylase, citrate cleavage enzyme and malic enzyme supporting the previous concept that cysteine supplementation is not the metabolic equivalent of methionine supplementation. In addition, when rats were fed diets with different levels of inorganic sulfate and supplemented with at least adequate cysteine, there was no decrease in citrate cleavage enzyme or malic enzyme activity greater than 30% when compared with the activity of those rats fed the diet optimal with respect to sulfate and no decrease in acetyl CoA carboxylase activity greater than 30%. Therefore, it appears that in livers from rats fed diets supplemented with cysteine that citrate cleavage enzyme and malic enzyme activity is sufficient to supply the acetyl CoA fragments and the reducing equivalents for fatty acid biosynthesis.

Previous observations have shown that the omission of methionine from the diet is not compensated by the addition of levels of inorganic sulfate above the optimum. The activity of acetyl CoA carboxylase is decreased when rats are fed a high level of inorganic sulfate (0.42%) and an inadequate level of methionine (table 8 page 64) as opposed to

a high sulfate level and an optimum level of methionine (table 9 page 66) demonstrating that at inorganic sulfate levels above the optimum there is no further sparing of methionine.

CHAPTER VI

SUMMARY

Metabolic alterations have been demonstrated to occur in rats fed diets containing inorganic sulfate at levels above or below the established optimal level of 0.02%. The level of dietary sulfate has been shown to influence certain key enzymes of carbohydrate metabolism. This investigation was designed to extend these studies to include lipogenesis by investigating the effect of varying the ratio and source of neutral and inorganic sulfur on the enzyme systems of acetyl CoA carboxylase, citrate cleavage enzyme and malic enzyme.

A decrease in the activities of acetyl CoA carboxylase and citrate cleavage enzyme and an increase in the activity of malic enzyme at inorganic sulfate levels above and below the otpimum were observed when diets containing constant levels of total sulfur as sulfate supplemented with inverse amounts of methionine and inorganic sulfate were fed. These findings are consistent with observations of the effect of high levels of inorganic sulfate in the diet increasing gluconeogenesis since a decrease in lipogenic enzyme activities would be expected when gluconeogenesis was increased. The effect on malic enzyme activity supports the suggestion that malic enzyme may have other metabolic functions besides that in lipogenesis.

When methionine supplementation was held constant, acetyl CoA carboxylase activity was increased and citrate cleavage enzyme activity was decreased when inorganic sulfate was fed at levels above or below the optimum. The effect observed with acetyl CoA carboxylase is

similar to previous findings with propionyl CoA carboxylase activity.

A decrease in the activity of acetyl CoA carboxylase with no methionine supplementation of the diet containing 0.42% $SO_4^=$ as compared to adequate methionine supplementation was observed. These findings reinforce previous observations that inorganic sulfate has some role in metabolism other than that of methionine sparing.

The substitutions of cysteine for methionine in the diets resulted in a decrease in the activities of acetyl CoA carboxylase, citrate cleavage enzyme, and malic enzyme. These findings demonstrate that cysteine is not the metabolic equivalent of methionine.

The data presented in this investigation show that both the level of neutral sulfur and inorganic sulfate may alter the apparent rate of lipogenesis and thus support the concept of inorganic sulfate as a metabolic regulator.

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VITA

Joyce Brann Bittle, daughter of Mr. and Mrs. Eugene Brann, was born in Jackson, Tennessee on April 11, 1942. She attended public schools in Jackson, Tennessee graduating from Jackson High School in 1960. In June 1964, the author was awarded the Bachelor of Science degree in education from The University of Tennessee, Knoxville. Her son, Fred L. Bittle, III, was born in Greeneville, Tennessee on January 4, 1968. The author entered Graduate School at The University of Tennessee in June 1973 and received the Master of Science degree in August 1975 and the Doctor of Philosophy degree in June 1977.