EFFECTS OF BIOTIN DEFICIENCY ON LIPOGENESIS AND CHOLESTEROGENESIS

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by
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To my wife

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ABSTRACT

Although acetyl CoA carboxylase (acetyl CoA:CO_2 ligase (ADP); EC 6.4.1.3) was the first to be recognized as a biotin enzyme, previous attempts to produce biotin deficiency effects on lipogenesis *in vivo* have not been successful. The effect of biotin deficiency on the lipid composition of liver, carcass and adipose tissue has been studied. Incorporation of acetate-1-^{14}C into liver lipids, liver acetyl CoA concentration and the *in vitro* activities of the mitochondrial and supernatant pathways of fatty acid synthesis were also investigated in both biotin deficient and pair-weighed control rats. Fat-free diets were used in these experiments. There was no significant difference in the total lipid content of liver between biotin deficient and control rats. However, deficient rat livers had significantly lower amounts of cholesterol esters and esterified fatty acids. The esterified fatty acid content of deficient carcasses was only 40% of the control level. In acetate-1-^{14}C incorporation experiments, specific activities of triglyceride, phospholipid and cholesterol ester fractions of the deficient rat liver were only 25% of those of controls. The increase in liver acetyl CoA concentration of deficient rats could not account for the large difference in the specific activities of the liver lipid components. Liver

acetyl CoA carboxylase activities of biotin deficient rats, per mg enzyme protein or per g fresh liver approximated 50% of that of controls. On the other hand, mitochondria from deficient rat livers had only 30% of the fatty acid synthetic activity of control mitochondria. The most significant effect of biotin deficiency was noted in the adipose tissue. The epididymal fat pad weight, total lipid, triglyceride, free fatty acid and phospholipid content of deficient rats were respectively 40, 35, 25, 20 and 25% of control values. These results indicate that the adipose tissue might quantitatively be more significant in lipogenesis and that biotin deficiency might have a more drastic effect on the metabolism of the adipose tissue than on liver.

ABBREVIATIONS

Tris	:	tris(hydroxymethyl)aminomethane
ATP	:	adenosine triphosphate
ADP	:	adenosine diphosphate
CoA	:	coenzyme A
PPO	:	2,5-diphenyloxazole
POPOP	:	1,4-bis-2(5-phenyloxazolyl)-benzene
TCA	:	trichloroacetic acid
NAD	:	nicotinamide adenine dinucleotide
NADH	:	nicotinamide adenine dinucleotide, reduced
NADP	:	nicotinamide adenine dinucleotide phosphate
NADPH	:	nicotinamide adenine dinucleotide phosphate, reduced
P_i	:	inorganic phosphate
EDTA	:	ethylenediamine tetra-acetate
GSH	:	glutathione
HMGCoA	:	β-hydroxy-β-methylglutaryl CoA

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SECTION I. INTRODUCTION

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A) Purpose

Although acetyl CoA carboxylase was the first to be recognized (1) as a biotin enzyme, previous attempts (2, 3) to study the effect of biotin deficiency on lipogenesis *in vivo* have not been entirely successful. Thus, Donaldson (2) found that the incorporation of acetate-1-^{14}C into the saponifiable fraction of liver lipids was not significantly decreased in biotin deficient chicks as compared to biotin treated controls. The incorporation into carcass saponifiable fraction of deficient chicks was lower than in controls. Puddu *et al*. (3) did not find any significant difference between normal and biotin deficient rats either in total lipid content or lipid composition of the liver. Suomalainen and Keranen (4) found that when baker's yeast was grown aerobically in absence of biotin there was a reduction in C_{18} fatty acids but an increase of fatty acids with 16 carbon atoms or less. There have been many contradictory reports regarding the participation of biotin in cholesterol synthesis (5-8). The effect of biotin deficiency on lipogenesis *in vivo* has been investigated. Its effect on the synthesis of various lipid components has also been studied.

B) General Approach

The effects of two deficient diets on cholesterol levels in serum, liver and carcass were first studied. Diet A was a high carbohydrate (66%) diet and diet B was a low carbohydrate (36%) diet. Both diets had the same fat content (5%). It was assumed that cholesterol synthesis on diet B would be contributed to a great extent by leucine of the dietary protein. Cholesterol synthesis from leucine is known to require a biotin enzyme, β-methylcrotonyl coenzyme A carboxylase (3-methylcrotonyl CoA:CO_2 ligase (ADP); EC 6.4.1.4). Hence, any reduction in cholesterol synthesis in biotin deficiency might be exaggerated by this altered dietary pattern where leucine rather than acetyl CoA would be the major precursor.

From this comparative study it was decided to use diet A in further work, with one modification; that the diet was made fat-free (diet C), since fat feeding has been reported to inhibit lipogenesis (9). This would mask any effects of biotin deficiency on lipid metabolism. Diet D, a low fat diet, otherwise identical to diet A, was also studied. This diet, which contained linoleic acid and corn oil, served as a comparison to diet C.

The total lipid and lipid component levels were determined in liver, carcass and epididymal fat pads to study

the effects of the deficiency.

Acetate-1-^{14}C incorporation into liver lipids was also studied. Changes in the rate of synthesis of lipids or their components should be reflected in the rate of incorporation of the label in short term incorporation studies.

Liver acetyl CoA and acetoacetate concentrations were determined in the biotin deficient rat and compared with the controls. This was undertaken to see if the acetyl CoA pool size could cause an isotope dilution effect in acetate-1-^{14}C incorporation.

The effects of the deficiency on the two major fatty acid synthesizing pathways in the liver were studied.

C) Organization of the Thesis

Three main sections, Literature Review, Experimental and Discussion comprise the body of the thesis. The Literature Review includes: the discovery of biotin, the metabolic effects of biotin deficiency, the metabolic role of biotin and the effects of biotin deficiency on lipogenesis and cholesterogenesis. The Experimental section is divided into two parts. The first part comprises the methods and is subdivided into: production of biotin deficiency, biochemical criterion of biotin deficiency, lipid distribution studies, acetate-1-^{14}C incorporation,

determination of acetyl CoA and acetoacetate, *in vitro* mitochondrial fatty acid synthesizing system and *in vitro* non mitochondrial fatty acid synthesizing system. The second part of the Experimental section presents the results of the above experiments. The Discussion tries to correlate both *in vivo* and *in vitro* results to explain the effects of biotin deficiency on fatty acid and cholesterol syntheses respectively.

SECTION II. LITERATURE REVIEW

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A) Discovery of Biotin

Since the year 1901 when Wildiers (10) first described the stimulating effects of small amounts of organic material on the growth of yeast, the name "bios" has been given to the substance or substances causing the increased growth of yeast. In later years, bios was shown to be multiple in nature and was fractionated into bios I, IIa, IIb, etc.

Bios IIb attracted the attention of Kögl (11) who announced the isolation from egg yolk of minute amounts of a crystalline compound possessing the greatest part of the yeast activity of the bios IIb fraction. This compound was called "biotin" by Kögl.

In 1933, Allison, Hoover and Burk (12) described the growth and respiration-promoting effects of extracts from various sources for *Rhizobium trifolii*, a legume nodule organism. The active agent was named "coenzyme R". In 1939, West and Wilson (13) pointed out the similarity between the two and Nilsson, Bjälfve and Burström (14) found that a sample of Kögl's crystalline biotin possessed coenzyme R activity. It appeared that coenzyme R was identical with biotin.

Boas in 1927 described the effects produced in rats when large amounts of dried egg white were added to the diet (15). On such a regime the animals gradually lost their hair.

Dermatitis, skin hemorrhages and loss of body weight occurred, a spasticity developed and death ultimately resulted. Parsons and associate (16) studied extensively the egg white injury factor and the distribution of the protective factor found by Boas. György investigated the chemical and physical properties of the protective factor, which he called "vitamin H", and early in 1940, György *et al*. (17) suggested the possible identity of vitamin H with biotin and coenzyme R.

Since these early discoveries of biotin, it has been found in animal and plant tissues also and occurs mainly in combined forms. One of these biotin complexes is biocytin (ϵ-N-biotinyl-L-lysine), isolated from yeast by Wright *et al*. (18). Another complex, whose structure has not been elucidated, is the "soluble bound biotin", extracted from the peptic digests of swine liver. Both of these complexes are degraded to biotin by an enzyme believed to be a peptidase (19). At least two distinct liver protein fractions containing biotin have been described; these biotin-containing proteins have been termed "biotoproteins" (20).

B) Metabolic Effects of Biotin Deficiency

Biotin is the simplest of the naturally occurring compounds that counteract the nutritional deficiency induced in animals (including man) by the feeding of raw egg white. The toxic material in egg is a protein (avidin) with which

biotin combines, in stoichiometric proportions, to form an avidin-biotin complex (21). This complex is not dissociable except by heat treatment or acid hydrolysis, nor is it split by the enzymes of the gastrointestinal tract of higher animals (22). Hence the feeding of avidin can result in a biotin deficiency caused by the formation of the nondigestible complex within the intestinal tract.

Biotin deficiency is not normally encountered in man or even in laboratory animals kept on apparently biotin-free diets. This is a reflection of the ability of intestinal bacteria to synthesize sufficient biotin to meet the requirements of the host organism. Consequently, biotin deficiency is usually induced by the administration of avidin (or raw egg white) or by elimination of intestinal bacteria by a bacteriostatic agent. The symptoms of this deficiency have been described earlier.

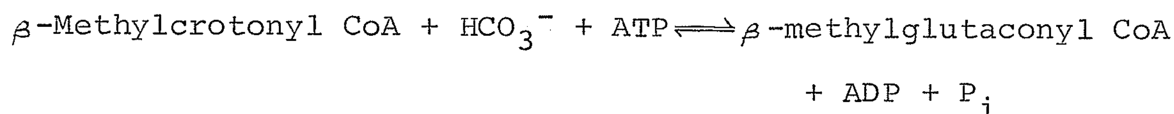
C) Metabolic Role of Biotin

Previously, biotin was thought to have a role of a coenzyme in certain carbon dioxide fixation reactions. Wakil *et al*. (1) found that purified preparations of acetyl CoA carboxylase contained biotin as a prosthetic group. Since then, five biotin enzymes have been characterized.

Acetyl CoA carboxylase, a biotin enzyme, is discussed further on in its role in fatty acid synthesis. The four

other biotin enzymes are: 1) β-methylcrotonyl CoA carboxylase (3-methylcrotonyl CoA:CO_2 ligase (ADP); EC 6.4.1.4), 2) propionyl CoA carboxylase (propionyl CoA:CO_2 ligase (ADP); EC 6.4.1.3), 3) methylmalonyl-oxalacetic transcarboxylase (methylmalonyl CoA:pyruvate carboxytransferase; EC 2.1.3.1), 4) pyruvate carboxylase (pyruvate:CO_2 ligase (ADP); EC 6.4.1.1).

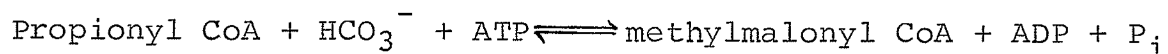
1) β-Methylcrotonyl CoA carboxylase. In the metabolism of isovaleryl coenzyme A which is on the pathway of leucine degradation, the isopropyl portion of the molecule is converted as a unit to acetoacetate by fixation of carbon dioxide. The reaction catalyzed is as follows:



$$\beta\text{-Methylcrotonyl CoA} + HCO_3^- + \text{ATP} \rightleftharpoons \beta\text{-methylglutaconyl CoA} + \text{ADP} + P_i$$

Fischer (23) showed that mitochondria from biotin deficient rat livers failed to oxidize isovalerate, whereas normal mitochondria oxidized it to acetoacetate. Furthermore, biotin was shown to be an integral part of the enzyme and purified preparations contained as much as 1 mole of biotin per 344,000 g of protein (24, 25).

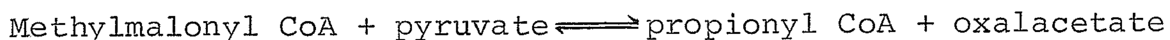
2) Propionyl CoA carboxylase. The following reaction is catalyzed by this enzyme:



$$\text{Propionyl CoA} + HCO_3^- + \text{ATP} \rightleftharpoons \text{methylmalonyl CoA} + \text{ADP} + P_i$$

Propionyl CoA carboxylase catalyzes the first step in the synthesis of succinyl CoA, since methylmalonyl CoA isomerizes to succinyl CoA. Lardy and Adler (26) showed that mitochondria from biotin deficient rats carboxylated propionate at a greatly reduced rate compared to mitochondria from normal animals. Kaziro _et al_. (27) found that the crystalline carboxylase had a molecular weight of 700,000 and contained 1 mole of biotin per 175,000 g of protein. Kosow and Lane (28) have shown that after eleven days on a biotin deficient diet there was a marked decrease of the carboxylase activity. Propionyl CoA carboxylase activity is a useful criterion for evaluating the biotin status of the animals as shown by Halenz and Lane (29).

3) Methylmalonyl-oxalacetic transcarboxylase. The enzyme catalyzes the following reaction:

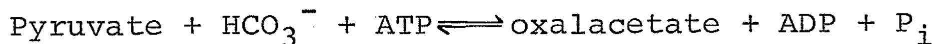


Methylmalonyl CoA + pyruvate $\rightleftharpoons$ propionyl CoA + oxalacetate

The role of biotin in the transcarboxylase reaction was established by Swick and Wood (30). An unusual feature of the methylmalonyl-oxalacetic transcarboxylase is that it catalyzes a transformation which involves compounds from different pathways, so shuttling carboxyl groups from one metabolic pathway to another for synthetic processes. This enzyme though is essentially a bacterial enzyme.

4) Pyruvate carboxylase. Utter and Keech (31) showed

conclusively the evidence for the occurrence in avian liver of an enzyme system which catalyzes the following reaction:



$$\text{Pyruvate} + HCO_3^- + \text{ATP} \rightleftharpoons \text{oxalacetate} + \text{ADP} + P_i$$

Keech and Utter (32) showed that avidin completely inhibited pyruvate carboxylase and Utter and Keech (31) also showed that it contained substantial amounts of biotin.

Thus biotin plays a direct role in five metabolic reactions, not as a cofactor but as an integral part of the enzyme catalyzing these reactions. However, the effects of biotin deficiency are felt in very many reactions in the intact organism. Biotin has been implicated in protein synthesis (33 - 36). Biotin has also been suggested to have an effect on carbohydrate metabolism. Dakshinamurti and Mistry (37) have shown that biotin deficiency results in increased incorporation of glucose-6-^{14}C and glucose-1-^{14}C into $^{14}CO_2$ as compared to pair-fed normals. Dakshinamurti and Cheah-Tan (38) also showed that glucose phosphorylation was decreased in biotin deficient rats when compared to pair-fed and pair-weighed controls.

Many effects of biotin deficiency have been noted, some direct and some indirect. In many cases the role of biotin is still obscure.

D) Role of Biotin in Fatty Acid Synthesis

The data from earlier studies concerning the participation of biotin in the synthesis of fatty acids were contradictory. Okey *et al*. (39) suggested that biotin was necessary for the synthesis and storage of fatty acids when they observed that the total fatty acid content of rat liver triples when the animals received a diet containing an excess of cholesterol, and that this did not occur when the cholesterol was administered to rats deficient in biotin. It should be noted that in this study, fatty acid values were total content per organ, and that the controls were fed *ad libitum* and consequently had larger livers. The differences would not have been so significant on a fatty acid content per unit weight of the organ as was done by Guggenheim and Olson (5) who disagreed with the above results. They showed that the total fatty acid level was more or less identical in the liver, heart and blood of biotin deficient rats in comparison with both pair-fed and *ad libitum* control groups. In this same paper, Guggenheim and Olson reported that the rate of incorporation of acetate-1-^{14}C into fatty acids revealed a normal capacity of biotin deficient rats to synthesize fatty acids.

Curran (40) reported similar findings to those of Guggenheim and Olson (5). He reported that in biotin

deficient rats the rate of fatty acid synthesis was slightly increased, but that this was due to the inanition accompanying biotin deficiency and not biotin deficiency itself. These observations as they pertain to fatty acid synthesis are indirect as in this work the distribution of deuterium oxide was the criterion used. Also the dose of administered deuterated water was not mentioned.

Wakil et al. (1) in studies with highly purified pigeon liver extracts showed that biotin was involved in a new enzymatic system for the synthesis of long chain fatty acids from acetyl CoA. Wakil et al. (41) isolated two main enzyme fractions, R_{1g} and R_{2g}, from pigeon liver supernatant. Fraction R_{1g} contained between 200 and 250 mμg of biotin per mg of protein or about 1 mole of biotin per 1×10^6 g of protein. This was the highest ratio between biotin and protein yet reported (1). The enzyme was later identified as acetyl CoA carboxylase (acetyl CoA:CO_2 ligase (ADP); EC 6.4.1.2). Support for the implication of biotin in this synthesis reaction came from the fact that the conversion of acetyl CoA to palmitate was inhibited in the presence of avidin and this inhibition was relieved by a supplement of biotin (1).

Bortz et al. (9) reported a feedback inhibition at the acetyl CoA carboxylase step brought about by fat feeding. They showed that after administration of 2 ml corn oil by

stomach tube the *in vitro* conversion of acetate to fatty acids was depressed as early as two hours after enteral administration to rats previously fed a fat-free, high carbohydrate diet. The depression was most pronounced at four hours after administration of the corn oil. They also reported that the conversion of malonate to fatty acids *in vitro* was not depressed by fat feeding.

Attempts (5, 40) to produce biotin effects on lipogenesis *in vivo* have not been successful, perhaps because the diets used contained 5-10% fat which itself would depress lipogenesis.

Donaldson (2), using a fat-free diet, showed that biotin deficiency in chicks resulted in increased incorporation of acetate-1-^{14}C into respiratory CO_2 and decreased incorporation into carcass fatty acids. He also showed that the deficiency had no effect on incorporation of malonate-2-^{14}C into respiratory CO_2 or liver and carcass lipids, and that the proportions of palmitate to stearate in the carcass were increased by biotin deficiency. The mechanism responsible for the decrease in stearate in biotin deficiency is still not known.

Puddu *et al*. (3), using a biotin deficient diet containing 5% fat, reported that no significant difference was found either in total lipid content or in lipid composition in the liver of the deficient rats when compared