6-2012

Identification and Assessment of Markers of Biotin Status in Healthy Adults

Wei Kay Eng
University of Nebraska – Lincoln, weikay@huskers.unl.edu

IDENTIFICATION AND ASSESSMENT OF MARKERS OF BIOTIN STATUS IN HEALTHY ADULTS

by

Wei Kay Eng

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Interdepartmental Area of Nutrition

Under the Supervision of Professor Janos Zempleni

Lincoln, Nebraska

June, 2012

IDENTIFICATION AND ASSESSMENT OF MARKERS OF BIOTIN STATUS IN HEALTHY ADULTS

Wei Kay Eng, M.S.

University of Nebraska, 2012

Advisor: Janos Zempleni

Human biotin requirements are unknown and the identification of reliable markers of biotin status is necessary to fill this knowledge gap. Here we used an outpatient feeding protocol to create states of biotin deficiency, sufficiency, and supplementation in 16 healthy men and women. Twenty possible markers of biotin status were assessed including the abundance of biotinylated carboxylases in lymphocytes, the expression of genes from biotin metabolism, and the urinary excretion of biotin and organic acids. Only the abundance of biotinylated 3-methylcrotonyl-CoA carboxylase (holo-MCC) and propionyl-CoA carboxylase (holo-PCC) allowed for distinguishing among all three levels of biotin intake. The urinary excretion of biotin reliably identified biotin-supplemented subjects, but did not distinguish between biotin-depleted and biotin-sufficient individuals. The urinary excretion of 3-hydroxyisovaleric acid (3-HIA) detected some biotin-deficient subjects, but produced a meaningful number of false negative results and did not distinguish between biotin-sufficient and biotin-supplemented individuals. The urinary excretion of 3-HIA might be a more reliable marker of biotin status, if used in combination with urinary citrate (positively associated with biotin intake) and malate (negatively associated with biotin intake). None of the other organic acids that were tested were useful markers of biotin status. Likewise, the abundance of mRNA coding for

biotin transporters, holocarboxylase synthetase, and biotin-dependent carboxylases in lymphocytes were not different among treatment groups. Generally, data sets were characterized by variations that exceeded those seen in studies in cell cultures. We conclude that holo-MCC and holo-PCC are the most reliable, single markers of biotin status tested in this study.

ACKNOWLEDGEMENTS

The journey of accomplishing this thesis contains hard work, frustration, excitement, and generous help from many people. I would like to take this opportunity to thank every individual who assisted, encouraged, and supported me during my program of studies.

First of all, I would like to thank my respectable advisor, Dr. Janos Zempleni for giving me an opportunity to join his lab. Without his guidance and support, both morally and financially, the completion of my research work would not be possible. I appreciate his time and effort in assisting me for making this research and thesis a success. I would like to extend my sincere gratitude to committee members, Dr. Schlegel and Dr. Cupp for their suggestions, advice, and support throughout the research phase of this thesis.

Secondly, I would like to thank my mentor, Dr. Samudra Wijeratne, and all my lab members, for their moral support and valuable suggestions to make this thesis a success. Special thanks are extended to David Giraud, our lab technician for his patience and willingness to guide me when I first entered the lab. Without his help, it would have been difficult to reach my research goals.

Last but not least, I would like to thank my family for believing and trusting me to do things on my own, for helping me when I couldn't, and for sending constant love and support across the world throughout my education. Your unconditional love, encouragement and support, made these achievements possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Page

CHAPTER 1

LITERATURE REVIEW

Biotin

Biotin, also known as vitamin B7 or H, is a water-soluble vitamin. In 1901, biotin was first discovered as a growth factor for yeast and Wildiers named it *bios*[33,61]. Boas determined that certain foods, such as yeast and liver, contained an organic substance that could prevent egg white toxicity[33,67]; an experiment conducted by Bateman in 1916 where he fed raw egg white to animals[33]. In 1931, Gyorgy discovered the organic substance in the liver and named it vitamin H[33,61]. In 1936, Kogl and Tonnis successfully isolated the crystalline growth factor from dried egg yolk and then called it biotin[32,33,47,61,67]. In 1941, Gyorgy and his associates also succeed in isolating biotin from the liver[61]. The chemical structure of biotin was determined by Vincent Du Vigneaud and colleagues in 1942[32,33,61]. The structure of biotin is formed from a cyclic urea structure with sulfur present in a thioether linkage, and carboxyl group present in a valeric acid side chain that is attached to the thioether linkage (Figure 1.1). This suggested that the structure of biotin is expressed as the chemical formula, $C_{10}H_{15}O_3N_2S$[15,38]. A year later, the vitamin was chemically synthesized by Harris et al. and available for experimentation[61].

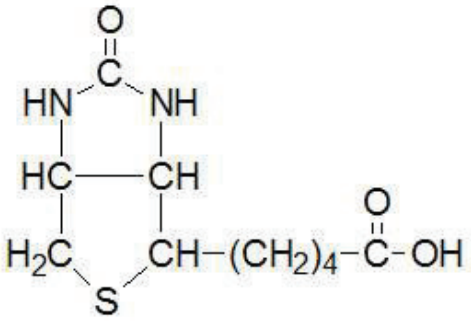

Figure 1.1. Structure of biotin

Biotin is an essential vitamin that needs to be consumed from food sources as humans cannot synthesize biotin. The Food and Nutrition Board of the National Research Council recommended the Adequate Intake (AI) of biotin to be 30 µg per day for adults. Biotin can be obtained through consuming a wide range of food like egg yolk, liver and some vegetables[71]. Large quantities of biotin are also synthesized in molds, yeasts and bacteria. Iron, the active ingredient in yeasts plays a crucial role in the biosynthesis of biotin. Biotin is an important coenzyme for five carboxylases which play important roles in gluconeogenesis, fatty acid synthesis and branched-chain amino acid catabolism[24]. Other than these, the biological functions of biotin include cell signaling, gene expression and chromatin structure[67].

Biotin Digestion and Transportation

The proteins that play major roles in biotin digestion and transportation are shown in Figure 1.2. The sources of biotin found in the body are protein-bound biotin in food, biotin-containing carboxylases, bacterial sources and free biotin[71]. In order to uptake free biotin into the small intestine epithelial cells, the protein-bound biotin in food needs to be digested by gastrointestinal proteases and peptidases into biocytin. Then, biotinidase (BTD) further break down biocytin into free biotin ready for absorption in the small intestine. The transport of free biotin across the brush-border membrane of the small intestine via Na^+-dependent carrier-mediated process or biotin-Na^+ co-transport system requires energy[49,51,52,67]. This occurs when biotin is in low concentration. However, at high extracellular concentration, biotin gets into cells through simple diffusion. At the intestinal basolateral membrane, biotin is transported via the carrier-

mediated process, but the system is Na^+-independent[53]. Bacterial sources of biotin synthesized in the large intestine released into the lumen of intestine have the same uptake mechanism as small intestine through Na^+-dependent carrier-mediated process[49]. The uptake of biotin into small and large intestine is affected by pantothenic acid and lipoic acid, which are believed to share the same transporter[49]. The name sodium dependent multivitamin transporter (SMVT) was given to this transporter for this reason[24,67].

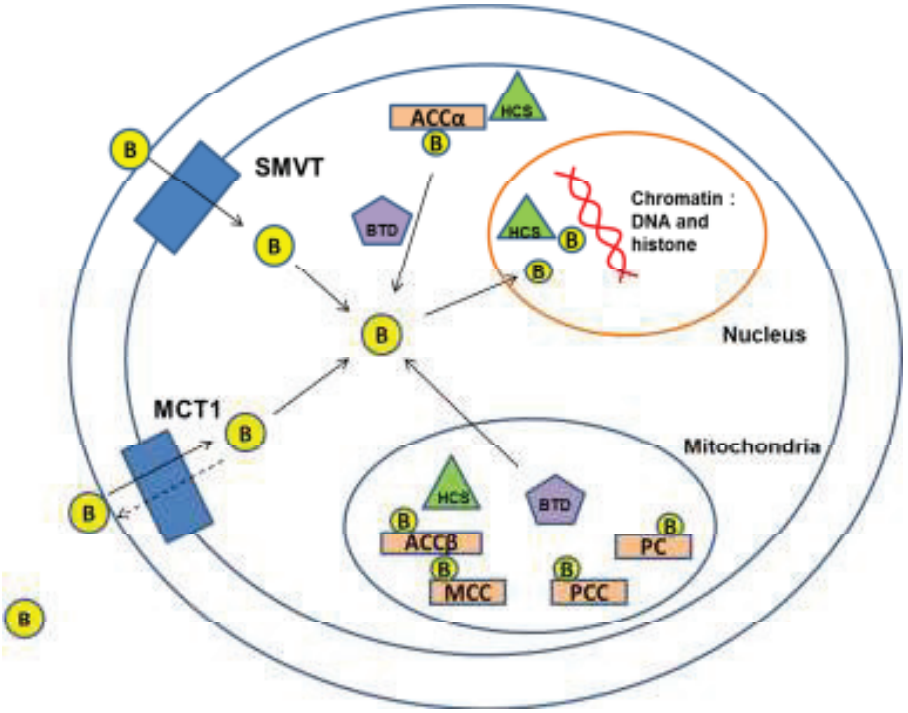

Figure 1.2. Transport mechanism of biotin transporters and localization of five biotin-dependent carboxylases in human cell. SMVT: sodium multivitamin transporter; MCTI: monocarboxylate transporter 1; B: biotin; PCC: propionyl-CoA carboxylase; ACCα: cytoplasmatic acetyl-CoA carboxylase α; ACCβ: mitochondrial acetyl-CoA carboxylase β; PC: pyruvate carboxylase; MCC: 3-methylcrotonyl-CoA carboxylase; HCS: holocarboxylasesynthetase, and BTD: biotinidase. **Modified from:** Zempleni J, Hassan YI & Wijeratne SS (2008) Biotin and biotinidase deficiency. *Expert Rev. Endocrinol. Metab.* **3**, 715-724.

Prasad et al. cloned SMVT from human intestinal cell line Caco-2. The Caco-2 SMVT cDNA codes for a protein of 635 amino acids, which is found to be homologous to the SMVT cloned from rat and rabbit intestines[50,51]. The knock down of hSMVT system of the Caco-2 cells leads to the decrease in hSMVT mRNA and protein level, and inhibits carrier-mediated biotin uptake[45]. The rat placental cDNA encoding SMVT was isolated and expressed in HeLa cells. It induces Na^+-dependent pantothenate and biotin transport activities which have been shown to be identical to the human placental choriocarcinoma cell line (JAR)[64]. This suggested that placenta required SMVT to transport nutrients from mother to fetus. SMVT (product of SLC5A6 gene) is located at chromosome 2p23. It is about 14 kilobase pairs in length and consists of 17 exons. Two distinct promoters and their potential transcriptional start sites have been found at −4603, and −4303. Both promoter sequences lack TATA and CAAT element, contain GC-rich sites and have multiple putative regulatory *cis*-elements, for instance, AP-1, AP-2, C/EBP, SP1, NF1, and GATA. The minimal region required for basal activity of the human SMVT promoter have been determined to be encoded by a sequence between −5846 and −5313 for promoter 1 and between −4417 and −4244 for promoter 2[14].

SMVT is an important biotin transporter for the kidney and intestine but few studies showed that SMVT is not the only transporter available for the uptake of biotin. One study showed that pantothenic acid and lipoic acid, which are substrates to SMVT did not affect the uptake of biotin in peripheral blood mononuclear cells (PBMC)[70,72]. It was determined that a 3 year old child was biotin deficient due to an inborn error in biotin transport. However, it did not affect the uptake of pantothenic acid in the PBMC[36]. The discovery of monocarboxylate transporter 1 (MCT1) solved the questions brought forth

in these studies. MCT1 mediates biotin transport in human lymphoid cells. In the presence of MCT1 substrates (biotin competitors) and MCT inhibitors, the transport rate of biotin decreased, whereas the overexpression of MCT1 caused a 50 % increase in biotin uptake. However, it did not affect biotin uptake in non-lymphoid cell lines suggesting a role of MCT1 as biotin transporter in human lymphoid cells[4].

Holocarboxylase Synthetase

Holocarboxylase synthetase (HLCS) is an enzyme that mediates the binding of biotin to the lysine residue of human apo-carboxylases and histones[19]. The similar protein present in the prokaryotes is known as BirA[6]. The human HLCS (hHLCS) catalyzes the biotinylation of five mammalian biotin-dependent carboxylases: propionyl-CoA carboxylase (PCC), cytoplasmic acetyl-CoA carboxylase 1 (ACC1), mitochondrial acetyl-CoA carboxylase 2 (ACC2), pyruvate carboxylase (PC) and 3-methylcrotonyl-CoA carboxylase (MCC) that are responsible for metabolic activities. The biotinylation of histone by hHLCS affects the chromatin structure and plays a role in gene regulation[21]. HLCS is located at chromosome 21q22.1[62] and has been detected in cytoplasm, mitochondria, cell nuclei, and the nuclear lamina[19]. The full length human HLCS encodes 726 amino acids and three splicing variants are observed with the molecular weight of 76, 82 and 86 kDa[19,22]. HLCS comprises of four domains: the N-terminal domain, central domain, linker domain, and C-terminal domain. N- and C-termini participate in recognizing biotin as a substrate for histone- and carboxylase-biotinylation. The central domain contains binding sites for both ATP and biotin while linker domain is for HLCS to interact with other substrates for biotinylation. The binding

of biotin to lysine residue of carboxylases and histones requires two steps. The first step is the synthesis of intermediate, biotinyl-5′-AMP that requires ATP; second, the ligation of biotinyl moiety to the lysine residue[20].

With the role of HLCS as an enzyme catalyzing the transfer of biotin to apo-carboxylases, deficiency of HLCS can lead to multiple carboxylase deficiency (MCD), an autosomal recessive disorder that is life threatening. The symptoms of HLCS deficiency are metabolic acidosis, a characteristic organic aciduria, lethargy, hypotonia, convulsions, and dermatitis[63]. These symptoms are normally apparent in the newborn to early infantile period[63]. However, studies have shown that patients with this disease can be cured by administering pharmacological doses of biotin[56]. HLCS deficiency also leads to a decrease in biotin binding to histones which eventually affects gene regulations and chromatin structure. Camporeale et al. discovered that HLCS knockdown causes phenotypes such as decreased life span and decreased heat survival in *Drosophila melanogaster*[7].

Chromatin

Total length of DNA in human cells is almost 2 meters. In order to fit into a nucleus with 5 to 10 μm, DNA has to package into a small and organized structure known as chromatin. Chromatin comprises of repetitive units of nucleosomes which are composed of DNA (147 base pairs) wrapped around histones. There are two forms of chromatin: heterochromatin and euchromatin. Heterochromatin has the most compact form of DNA that encodes inactive genes. In contrast, euchromatin is a loosely packed form of chromatin that is rich in actively transcribed genes. Chromatin contacts with the

DNA can be disrupted and bonded by other proteins recruited for modifications on histones.

Histones

Histones are small proteins (11 to 22 kDa) that fold DNA into a more organized and condensed structure (chromatin) to be packaged in the nuclei[30]. Five major classes of histones have been identified in the eukaryotic cells: H1, H2A, H2B, H3 and H4[11]. These histones form a core octamer core histone (one H3-H3-H4-H4 tetramer and two H2A-H2B dimers) that is wrapped around with DNA to form the repetitive nucleosomal core particle[67]. Histone H1 is the linker histone that connects each core particle to complete the nucleosomal assembly[8]. The binding of DNA to histones is stabilized by electrostatic interactions between the association of negatively charged phosphate groups of DNA with positively charged amino groups (lysine residue) and guanidino groups (arginine residue) of histones that account for more than 20 % of all amino acids residues[30,67]. Histones consist of a globular C-terminal domain and a flexible N-terminal domain tail that protrudes from the nucleosome surface available for posttranslational modifications[8].

Covalent Modifications of Histones

The unstructured N-terminal tail of histones protruding from the nucleosome is a target for covalent modifications. Covalent modifications of histones are known to play a crucial role in gene transcriptional activity, DNA repair, and chromatin structure. The types of modifications found on histones are: acetylation, methylation, phosphorylation,

ubiquitylation, sumoylation, poly (ADP-ribosylation), and biotinylation (as discussed later)[31] (Figure 1.3). Among all these modifications, histone acetylation has been the most studied[58]. Histone acetylation plays a fundamental role in transcriptional regulation[58]. Histone acetyl transferase (HAT) catalyzes the acetylation of lysine on histones and can neutralize the basic charge of the lysine and eventually dissociates the DNA from histones[31]. This allows the RNA polymerase to bind to the promoter region for transcription to occur[31]. Studies have shown that phosphorylation of the serine 10 residue of the N-terminal tail of histone H3 is crucial for chromosome condensation and segregation during mitosis and meiosis[18,65]. However, a non-phosphorylable histone H3 showed abnormal chromosome segregation, resulting in chromosome loss during mitosis and abnormal chromosome condensation[18,42]. Methylation of histone might mark a gene to be or not be transcribed[74]. For instance, the methylation of lysine 9 (K9) on histone H3 contributes to gene silencing[2,17]. However, another example comes from yeast, where active promoters are trimethylated at K4 in histone H3, but when they are dimethylated, genes are repressed[2]. Evidence showed that poly (ADP-ribosylation) of histone is linked to DNA repair mechanism[1,44]. Shiio et al. provided evidence that histone sumoylation mediates gene silencing through recruitment of histone deacetylase and heterochromatin protein 1[55].

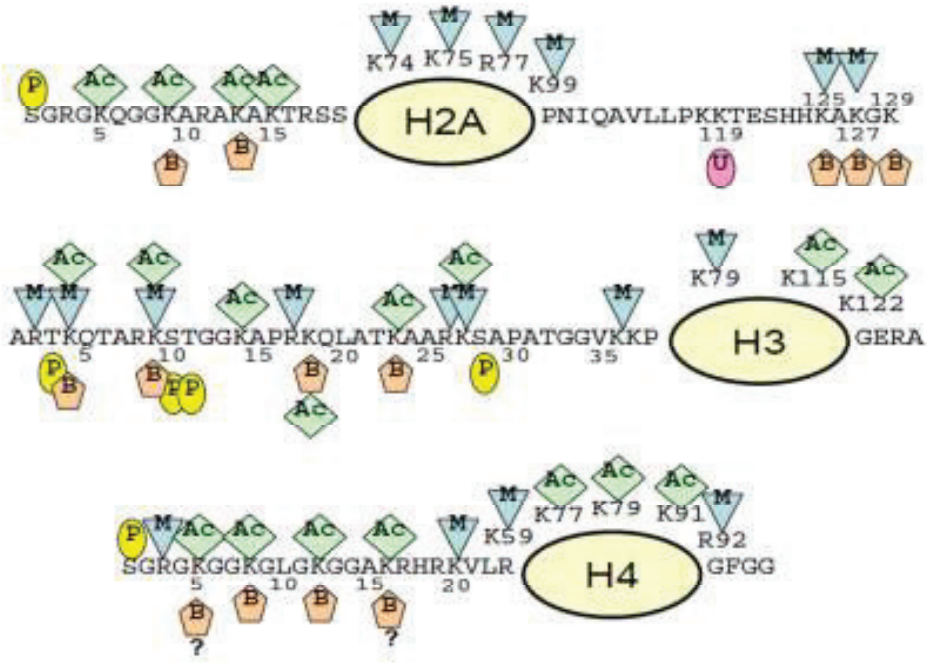

Figure 1.3. Covalent modification sites in histones.
Abbreviations: A: acetate, B: biotin, M: methyl, P: phosphate, U: ubiquitin.

Biotinylation of Histones

Histone biotinylation is one of the covalent modifications of histones that have been discovered recently. Biotinylation of histone is a naturally occurring phenomenon that was confirmed in many studies[57]. To date, eleven biotinylation sites on the histones have been identified using histone-based synthetic peptides and biotinylation site-specific antibodies[46]; K9, K13, K125, K127 and K129 in histone H2A; K4, K9, K18, and perhaps K23 in histone H3, and K8, K12 and K16 in histone H4[16,43]. The functional roles of histone biontinylation are gene silencing, cellular responses to DNA damage, and cell proliferation[73]. Initially, it was proposed by Wolf and co-workers that histone

biotinylation is catalyzed by BTD[23]. However, recent studies show that HLCS has histone biotinyl ligase activity[3].

Previous study reported that K12-biotinylated histone H4 (H4K12bio) is enriched in repeat regions such as pericentromeric alpha satellite repeats, telomeres, and long terminal repeats (LTR)[12,68]. Increased abundance of H4K12bio is associated with repression of transposons that in turn increases genome stability and decreases cancer risk[68]. In addition, biotinylation of lysine-9 in histone H2A (H2AK9bio) also represses retrotransposon transcription in human and mouse cell lines. The depletion of H2AK9bio showed increased production of viral particles that eventually leads to genome instability[12]. Similarly, H3K9bio, H3K18bio, and H4K8bio are also associated with heterochromatin and LTRs[43]. The enrichment of biotinylation marks in histones and their gene repression effects depend on biotin status, as shown in cell culture- and human- studies[27]. In Jurkat cells, increased histone biotinylation was observed in all classes of histones after exposure to UV light suggesting that increased biotinylation of histones in DNA-damaged cells might be a step during apoptosis[44].

Apo-carboxylase and Holo-carboxylase

In mammals, biotin serves as a covalently bound coenzyme for five biotin-dependent carboxylases: PCC, cytoplasmic ACC1, mitochondrial ACC2, PC and MCC. HLCS catalyzes the transfer of biotin to these carboxylases. The procedure requires a two-step reaction. First, the activated intermediate, bio-5'-AMP, is synthesized from biotin and ATP, followed by covalent linkage of the biotin moiety to the amino group of lysine residue in carboxylase[10,13,29,71]. Carboxylase without biotin attachment (inactive)

is known as apo-carboxylase, whereas the biotinylated carboxylase (active) is also known as holocarboxylase.

ACC1 resides in the cytosol and catalyzes the binding of bicarbonate to acetyl-CoA to form malonyl-CoA, the substrate of fatty acid synthesis. ACC2 is a product of a different gene but catalyzes the same reaction as ACC1. It is involved in controlling fatty acid oxidation in the mitochondria. The malonyl-CoA produced by ACC2 acts as an inhibitor for fatty acid transport to mitochondria. ACC2 also plays an important role in biotin storage. PCC catalyzes synthesis of S-methylmalonyl-CoA from propionyl-CoA, which is part of the odd-chain fatty acid synthesis pathway. PCC is also involved in isoleucine, threonine, methionine, and valine catabolism. PC is located in the mitochondrial matrix and it is a key enzyme in gluconeogenesis that is responsible for the carboxylation of pyruvate to oxaloacetate. Finally, mitochondrial MCC catalyzes an essential step in leucine metabolism by converting 3-methylcrotonoyl-CoA to 3-methylglutaconyl-CoA[13,34,66,71]. The biotin that binds to the carboxylases is recyclable. First, holocarboxylases (biotinylated carboxylases) will be degraded to form biotinylated peptides, e.g., biocytin. These peptides are further degraded by BTD to release free biotin, which can then be used for the synthesis of new holocarboxylases[35].

Biotin Catabolism

Two major pathways have been discovered by McCormick et al. in biotin catabolism[4,5,25,26,48](Figure 1.4). In the first pathway, the valeric acid side chain of biotin is catabolized by β-oxidation. The cleavage of two carbon units leads to the formation of bisnorbiotin. The continuous cleavage of two carbon units generates tetranorbiotin (one carbon unit). The heterocyclic ring of biotin molecule cleaves and degrades after the valeric acid side chain is degraded to one carbon unit. In the second pathway, sulfur on the heterocyclic ring of biotin molecule is oxidized, forming biotin-*l*-sulfoxide, biotin-*d*-sulfoxide and biotin sulfone. This pathway occurs in the smooth endoplasmic reticulum with the help of nicotinamide adenine dinucleotide phosphate (NADPH). The combinations of β-oxidation and sulfur oxidation also occur to generate bisnorbiotin sulfone[4,5,9,25,26,28,48]. The total concentration of these biotin catabolites approximately equals the actual biotin consumption that can be used to quantify biotin intake[67]. The urinary excretion of biotin metabolites is used as a marker to identify the biotin status of an individual.

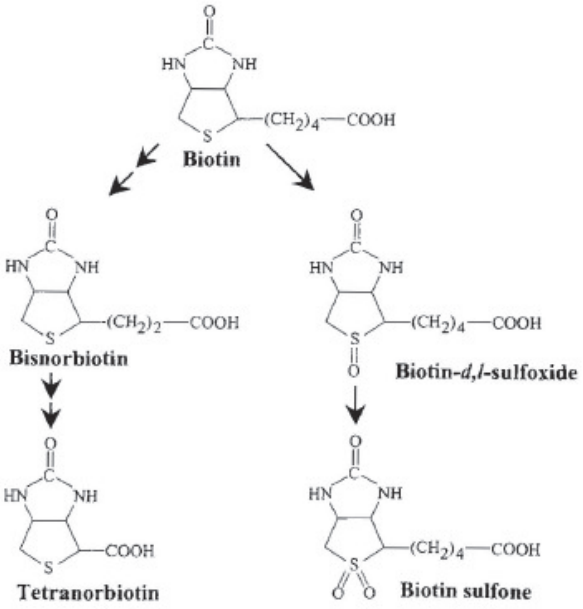

Figure 1.4. Biotin catabolism. Taken from: Camporeale G & Zempleni J (2006) Biotin. In *Present Knowledge in Nutrition*, 9th ed., pp. 314-326 [BA Bowman and RM Russell, editors]. Washington, D.C.: International Life Sciences Institute.

Biotin Deficiency

Although biotin sources can be found in wide range of food sources, biotin deficiency is not rare. Many clinical findings of biotin deficiency have been reported. The signs of biotin deficiency have been identified in patients with long term therapy with certain anticonvulsants, patients receiving parenteral nutrition without biotin supplementation, pregnant and lactating women, smokers, individuals with prolonged consumption of raw egg white as well as individuals with genetic inborn errors in metabolism[9]. The symptoms of biotin deficiency include seizures, hypotonia, ataxia, dermatitis, hair loss, mental retardation, ketolactic acidosis, organic aciduria and also

fetal malformations[69]. Fortunately, administration of pharmacological oral doses of biotin can reverse biotin deficiency.

Most of the cases in biotin deficiency have been assessed by quantifying the urinary excretion of biotin and its metabolites, activities of PCC and beta-MCC in lymphocytes, and urinary excretion of 3-HIA (Figure 1.5). Based on a study completed on pregnant women by Mock et al.[41], about half of the pregnant women appeared to become at least marginally biotin deficient late in pregnancy. The result showed increased urinary excretion of 3-HIA and decreased excretion of biotin in late pregnancy. Decreased excretion of biotin was due to increased biotin biotransformation into biotin catabolites during pregnancy[41]. The lack of biotin could lead to the impairment of MCC required for leucine metabolism. Decreased activity of MCC shunts the substrate 3-methylcrotonyl-CoA to an alternate pathway, resulting in the abnormal increased production of 3HIA, a promising organic acid in urine[39,59,60].

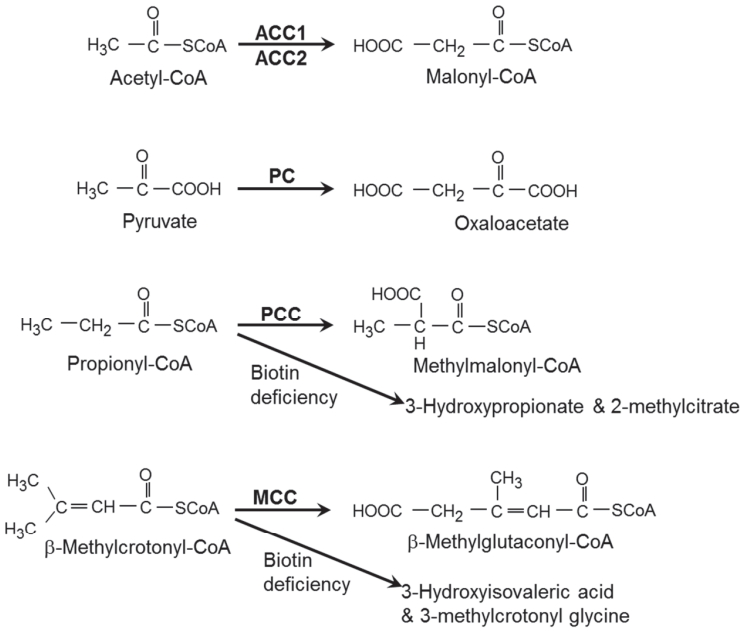

Figure 1.5. Biotin deficiency impairs metabolism. Taken from: Camporeale G & Zempleni J (2006) Biotin. In *Present Knowledge in Nutrition*, 9th ed., pp. 314-326 [BA Bowman and RM Russell, editors]. Washington, D.C.: International Life Sciences Institute

A study completed on a group of smokers in identifying their biotin status showed smoking accelerates biotin catabolism in women. The smoking women excreted significantly less urinary biotin. However, the ratio of urinary biotin metabolites (biotin sulfoxide) to biotin increased in these women suggesting that smoking degrades nutrients, one of them being biotin[54]. Also, long term use of anticonvulsants increases urinary excretion of 3HIA and biotin catabolism. These results were attributed to the ability of anticonvulsants to prevent biotin uptake into the brush-border membrane vesicles from intestine[71]. Prolonged consumption of raw egg white can lead to biotin deficiency

because it contains avidin, a protein that binds tightly to biotin thereby preventing its absorption into the intestine.

Biotin serves as a cofactor for five biotin-dependent carboxylases. The absence of biotin in diet could lead to the impairment of energy metabolism pathways. Biotin deficiency induced by an egg-white diet in rats decreased lymphocyte PCC activity[40]. These results were further supported by another study completed on Jurkat cells. PCC protein abundance decreased in cells cultured in biotin deficient media[27]. PC is a mitochondrial enzyme that catalyzes the conversion of pyruvate to oxaloacetate in the citric acid cycle for energy production. PC deficiency causes multiorgan metabolic imbalance that mainly exhibits with lactic acidemia and neurological dysfunction at an early age[37].

Biotin is also important for mediating gene regulation and genome stability. A study performed by Chew et al. showed that biotin deficiency leads to decreased biotinylation of histone i.e., H4K12bio, in the transposon elements[12]. This deficiency increased the risk of retrotransposon transcription, viral production and eventually genome instability[12].

.

REFERENCES

1. Althaus F (1992) Poly ADP-ribosylation: a histone shuttle mechanism in DNA excision repair. *J. Cell Sci.* **102**, 663-670.
2. Bannister AJ, Schneider R & Kouzarides T (2002) Histone methylation: dynamic or static? *Cell* **109**, 801-806.
3. Bao B, Pestinger V, I. HY *et al.* (2011) Holocarboxylase synthetase is a chromatin protein and interacts directly with histone H3 to mediate biotinylation of K9 and K18. *J. Nutr. Biochem.* **22**, 470-475.
4. Brady RN, Li L-F, McCormick DB *et al.* (1965) Bacterial and enzymatic degradation of biotin. *Biochem Biophys Res Commun* **19**, 777-782.
5. Brady RN, Ruis H, McCormick DB *et al.* (1966) Bacterial degradation of biotin. Catabolism of 14C-biotin and its sulfoxides. *J. Biol. Chem.* **241**, 4715-4721.
6. Campeau E & Gravel RA (2001) Expression in *Escherichia coli* of N- and C-terminally deleted human holocarboxylase synthetase. Influence of the N-terminus on biotinylation and identification of a minimum functional protein. *J. Biol. Chem.* **276**, 12310-12316.
7. Camporeale G, Giordano E, Rendina R *et al.* (2006) *Drosophila* holocarboxylase synthetase is a chromosomal protein required for normal histone biotinylation, gene transcription patterns, lifespan and heat tolerance. *J. Nutr.* **136**, 2735-2742.
8. Camporeale G, Shubert EE, Sarath G *et al.* (2004) K8 and K12 are biotinylated in human histone H4. *Eur. J. Biochem.* 271, 2257-2263.
9. Camporeale G & Zempleni J (2006) Biotin. In *Present Knowledge in Nutrition*, 9th ed., pp. 314-326 [BA Bowman and RM Russell, editors]. Washington, D.C.: International Life Sciences Institute.
10. Chapman-Smith A & Cronan JEJ (1999) Molecular Biology of Biotin Attachment to Proteins. *J. Nutr.* **129**, 477S-484S.
11. Chew YC, Camporeale G, Kothapalli N *et al.* (2006) Lysine residues in N- and C-terminal regions of human histone H2A are targets for biotinylation by biotinidase. *J. Nutr. Biochem.* **17**, 225-233.
12. Chew YC, West JT, Kratzer SJ *et al.* (2008) Biotinylation of histones represses transposable elements in human and mouse cells and cell lines, and in Drosophila melanogaster. *J. Nutr.* **138**, 2316-2322.
13. Daberkow RL, White BR, Cederberg RA *et al.* (2003) Monocarboxylate transporter 1 mediates biotin uptake in human peripheral blood mononuclear cells. *J. Nutr.* **133**, 2703-2706.
14. Dey S, Subramanian VS, Chatterjee NS *et al.* (2002) Characterization of the 5' regulatory region of the human sodium-dependent multivitamin transporter, hSMVT. *Biochim. Biophys. Acta* **1574**, 187-192.
15. du Vigneaud V, Melville DB, Folkers K *et al.* (1942) The structure of biotin: A study of desthiobiotin. *J. Biol. Chem.* **146**, 475-485.
16. Filenko NA, Kolar C, West JT *et al.* (2011) The role of histone H4 biotinylation in the structure and dynamics of nucleosomes. *PLoS ONE* **6**, e16299.
17. Fuks F (2005) DNA methylation and histone modifications: teaming up to silence genes. *Curr Opin Genet Dev* **15**, 490-495.

18. Hans F & Dimitrov S (2001) Histone H3 phosphorylation and cell division. *Oncogene* **20**, 3021-3027.
19. Hassan YI, Moriyama H, Olsen LJ *et al.* (2009) N- and C-terminal domains in human holocarboxylase synthetase participate in substrate recognition. *Mol. Genet. Metab.* **96**, 183-188.
20. Hassan YI, Moriyama H & Zempleni J (2010) The polypeptide Syn67 interacts physically with human holocarboxylase synthetase, but is not a target for biotinylation. *Archives of Biochemistry and Biophysics* **495**, 35-41.
21. Hassan YI & Zempleni J (2008) A novel, enigmatic histone modification: biotinylation of histones by holocarboxylase synthetase. *Nutr. Rev.* **66**, 721-725.
22. Hiratsuka M, Sakamoto O, Li X *et al.* (1998) Identification of holocarboxylase synthetase (HCS) proteins in human placenta. *Biochim. Biophys. Acta* **1385**, 165-171.
23. Hymes J, Fleischhauer K & Wolf B (1995) Biotinylation of histones by human serum biotinidase: assessment of biotinyl-transferase activity in sera from normal individuals and children with biotinidase deficiency. *Biochem. Mol. Med.* **56**, 76-83.
24. Hymes J & Wolf B (1999) Human biotinidase isn't just for recycling biotin. *J. Nutr.* **129**, 485S-489S.
25. Im WB, Roth JA, McCormick DB *et al.* (1970) Bacterial degradation of biotin: V. Metabolism of ^{14}C-carbonyl-labeled biotin *d*-sulfoxide. *J Biol Chem* **245**, 6269-6273.
26. Iwahara S, McCormick DB, Wright LD *et al.* (1969) Bacterial degradation of biotin. III. Metabolism of [^{14}C] carboxyl labeled biotin. *J Biol Chem* **244**, 1393-1398.
27. Kaur Mall G, Chew YC & Zempleni J (2010) Biotin requirements are lower in human Jurkat lymphoid cells but homeostatic mechanisms are similar to those of HepG2 liver cells. *J. Nutr.* **140**, 1086-1092.
28. Kazarinoff MN, Im W-B, Roth JA *et al.* (1972) Bacterial Degradation of Biotin VI. Isolation and Identification of b-hydroxy and b-keto compounds. *J Biol Chem* **247**, 75-83.
29. Knowles JR (1989) The mechanism of biotin-dependent enzymes. *Ann. Rev. Biochem.* **58**, 195-221.
30. Kobza K, Camporeale G, Rueckert B *et al.* (2005) K4, K9, and K18 in human histone H3 are targets for biotinylation by biotinidase. *FEBS J.* **272**, 4249-4259.
31. Kouzarides T (2007) Chromatin modifications and their function. *Cell* **128**, 693-705.
32. Lane MD (2004) The Biotin Connection: Severo Ochoa, Harland Wood, and Feodor Lynen. *J Biol Chem* **279**, 39187-39194.
33. Lardy HA & Peanasky R (1953) Metabolic Functions of Biotin. *Physiol Rev* **33**, 560-565.
34. Lynen F (1967) The role of biotin-dependent carboxylations in biosynthetic reactions. *Biochem J.* **102**, 381-400.
35. Manthey KC, Griffin JB & Zempleni J (2002) Biotin supply affects expression of biotin transporters, biotinylation of carboxylases, and metabolism of interleukin-2 in Jurkat cells. *J. Nutr.* **132**, 887-892.
36. Mardach R, Zempleni J, Wolf B *et al.* (2002) Biotin dependency due to a defect in biotin transport. *J. Clin. Invest.* **109**, 1617-1623.
37. Marin-Valencia I, Roe CR & Pascual JM (2010) Pyruvate carboxylase deficiency: mechanisms, mimics and anaplerosis. *Mol Genet Metab* **101**, 9-17.

38. Melville DB, Moyer AW, Hofmann K *et al.* (1942) The Structure of Biotin: The Formation of Thiophenevaleric Acid from Biotin. *J Biol Chem* **146**, 487-492.
39. Mock DM, Henrich CL, Carnell N *et al.* (2002) Indicators of marginal biotin deficiency and repletion in humans: validation of 3-hydroxyisovaleric acid excretion and a leucine challenge. *Am. J. Clin. Nutr.* **76**, 1061-1068.
40. Mock DM & Mock NI (2002) Lymphocyte propionyl-CoA carboxylase is an early and sensitive indicator of biotin deficiency in rats, but urinary excretion of 3-hydroxypropionic acid is not. *J. Nutr.* **132**, 1945-1950.
41. Mock DM, Stadler D, Stratton S *et al.* (1997) Biotin status assessed longitudinally in pregnant women. *J. Nutr.* **127**, 710-716.
42. Nowak SJ & Corces VG (2004) Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. *Trends Genet* **20**, 214-220.
43. Pestinger V, Wijeratne SSK, Rodriguez-Melendez R *et al.* (2011) Novel histone biotinylation marks are enriched in repeat regions and participate in repression of transcriptionally competent genes. *J. Nutr. Biochem.* **22**, 328-333.
44. Peters DM, Griffin JB, Stanley JS *et al.* (2002) Exposure to UV light causes increased biotinylation of histones in Jurkat cells. *Am. J. Physiol. Cell Physiol.* **283**, C878-C884.
45. Prasad P, Wang H, Huang W *et al.* (1999) Molecular and functional characterization of the intestinal Na^+-dependent multivitamin transporter. *Arch. Biochem. Biophys.* **366**, 95-106.
46. Rios-Avila L, Pestinger V & Zempleni J K16-biotinylated histone H4 is overrepresented in repeat regions and participates in the repression of transcriptionally competent genes in human Jurkat lymphoid cells. *J Nutr Biochem*.
47. Robbins WJ & Schmidt MB (1939) Preliminary Experiments on Biotin. *Bulletin of the Torrey Botanical Club* **66**, 139-150.
48. Roth JA, McCormick DB & Wright LD (1970) Bacterial degradation of biotin. IV. Metabolism of 14C-carbonyl-labeled biotin *l*-sulfoxide. *J Biol Chem* **245**, 6264-6268.
49. Said HM (2009) Cell and molecular aspects of human intestinal biotin absorption. *J. Nutr.* **139**, 158-162.
50. Said HM & Derweesh I (1991) Carrier-mediated mechanism for biotin transport in rabbit intestine - studies with brush-border membrane vesicles. *Am. J. Physiol.* **261**, R94-R97.
51. Said HM & Redha R (1987) A carrier-mediated system for transport of biotin in rat intestine in vitro. *Am. J. Physiol.* **252**, G52-G55.
52. Said HM, Redha R & Nylander W (1987) A carrier-mediated, Na+ gradient dependent transport for biotin in human intestinal brush border membrane vesicles. *Am. J. Physiol.* **253**, G631-G636.
53. Said HM, Redha R & Nylander W (1988) Biotin transport in basolateral membrane vesicles of human intestine. *Gastroenterology* **94**, 1157-1163.
54. Sealey WM, Teague AM, Stratton SL *et al.* (2004) Smoking accelerates biotin catabolism in women. *Am J Clin Nutr* **80**, 932-935.
55. Shiio Y & Eisenman RN (2003) Histone sumoylation is associated with transcriptional repression. *Proc. Natl. Acad. Sci. USA* **100**, 13225-13230.

56. Solorzano-Vargas RS, Pacheco-Alvarez D & Leon-Del-Rio A (2002) Holocarboxylase synthetase is an obligate participant in biotin-mediated regulation of its own expression and of biotin-dependent carboxylases mRNA levels in human cells. *Proc. Natl. Acad. Sci. USA* **99**, 5325-5330.
57. Stanley JS, Griffin JB, Mock DM *et al.* (2002) Biotin uptake into human peripheral blood mononuclear cells increases early in the cell cycle, increasing carboxylase activities. *J. Nutr.* **132**, 1854-1859.
58. Strahl BD & Allis CD (2000) The language of covalent histone modifications. *Nature* **403**, 41-45.
59. Stratton SL, Henrich CL, Matthews NI *et al.* (2012) Marginal Biotin Deficiency Can Be Induced Experimentally in Humans Using a Cost-Effective Outpatient Design. *J. Nutr.* **142**, 22-26.
60. Stratton SL, Horvath TD, Bogusiewicz A *et al.* (2011) Urinary excretion of 3-hydroxyisovaleryl carnitine is an early and sensitive indicator of marginal biotin deficiency in humans. *J. Nutr.* **141**, 353-358.
61. Streit WR & Entcheva P (2003) Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production. *Appl Microbiol Biotechnol* **61**, 21-31.
62. Suzuki Y, Aoki Y, Ishida Y *et al.* (1994) Isolation and characterization of mutations in the human holocarboxylase synthetase cDNA. *Nat. Genet.* **8**, 122-128.
63. Suzuki Y, Yang X, Aoki Y *et al.* (2005) Mutations in the holocarboxylase synthetase gene HLCS. *Human Mutation* **26**, 285-290.
64. Wang H, Huang W, Fei Y-J *et al.* (1999) Human placental Na^+-dependent multivitamin transporter. *J. Biol. Chem.* **274**, 14875-14883.
65. Wei Y, Yu L, Bowen J *et al.* (1999) Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. *Cell* **97**, 99-109.
66. Wolf B & Feldman GL (1982) The biotin-dependent carboxylase deficiencies. *Am. J. Hum. Genet.* **34**, 699-716.
67. Zempleni J (2005) Uptake, localization, and noncarboxylase roles of biotin. *Annu. Rev. Nutr.* **25**, 175-196.
68. Zempleni J, Chew YC, Bao B *et al.* (2009) Repression of transposable elements by histone biotinylation. *J. Nutr.* **139**, 2389-2392.
69. Zempleni J, Hassan YI & Wijeratne SS (2008) Biotin and biotinidase deficiency. *Expert Rev. Endocrinol. Metab.* **3**, 715-724.
70. Zempleni J & Mock DM (1998) Uptake and metabolism of biotin by human peripheral blood mononuclear cells. *Am. J. Physiol. Cell Physiol.* **275**, C382-C388.
71. Zempleni J & Mock DM (1999) Biotin biochemistry and human requirements. *J. Nutr. Biochem.* **10**, 128-138.
72. Zempleni J & Mock DM (1999) Human peripheral blood mononuclear cells: inhibition of biotin transport by reversible competition with pantothenic acid is quantitatively minor. *J. Nutr. Biochem.* **10**, 427-432.
73. Zempleni J, Wijeratne SS & Hassan YI (2009) Biotin. *Biofactors* **35**, 36-46.
74. Zhang Y & Reinberg D (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev* **15**, 2343-2360.

CHAPTER 2

IDENTIFICATION AND ASSESSMENT OF MARKERS OF BIOTIN STATUS IN HEALTHY ADULTS

IDENTIFICATION AND ASSESSMENT OF MARKERS OF BIOTIN STATUS IN HEALTHY ADULTS

Wei Kay Eng[1], David Giraud[1], Vicki L. Schlegel[2], Dong Wang[3], Bo Hyun Lee[2] and Janos Zempleni[1*]

[1]Departments of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0806, USA

[2]Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE 68583-0919, USA

[3]Statistics, University of Nebraska-Lincoln, Lincoln, NE 68583-0963, USA

Abbreviations: MCC, 3-methylcrotonyl-CoA carboxylase; PCC, propionyl-CoA carboxylase; ACC, acetyl-CoA carboxylases; PC, pyruvate carboxylase; HLCS, holocarboxylase synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SMVT, sodium-dependent multivitamin transporter; MCT1, monocarboxylate transporter 1; 3-HIA, 3-hydroxyisovaleric acid; HABA, 2-(4'hydroxyazobenzene)-benzoic acid.

*Corresponding author: Janos Zempleni, fax +1 402 472 1587, email jzempleni2@unl.edu

INTRODUCTION

Biotin is a water-soluble vitamin that serves as a coenzyme for five human carboxylases, namely acetyl-CoA carboxylases (ACC) 1 and 2, pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC), and 3-methylcrotonyl-CoA carboxylase (MCC)[50]. These enzymes play an important role in the metabolism of fatty acids, glucose, and amino acids. Histones H3 and H4 are also modified by covalent attachment of biotin[5,15,32,34]. Biotinylation of histones is a rare event and less than 0.001 % of histones H3 and H4 are biotinylated[1,16,39]. Biotinylated histones are overrepresented in repressed loci[4,32,34] and, despite their rarity, biotinylation marks contribute to chromatin condensation[8]. The binding of biotin to both carboxylases and histones is catalyzed by holocarboxylase synthetase (HLCS)[2,50].

Despite the essential roles of biotin in intermediary metabolism and gene regulation, human biotin requirements are unknown. Consequently, only recommendations for Adequate Intake, but no Recommended Dietary Allowance is available for biotin[30]. Recommendations for Adequate Intake are based solely on biotin intake in the general, apparently healthy, population[30]. This approach is flawed in the case of biotin, where dietary intake data are only crude estimates. Currently, no studies are available quantifying biotin in foods using chemically specific assays[48], and it is unclear whether intake estimates exceed or underestimate the true biotin intake. Also, the "normal state" is defined using biotin-dependent enzymes or urinary biotin metabolites as markers, while ignoring the changes occurring at the chromatin level.

As of today, no comprehensive study has been published that investigated markers of human biotin status from various possible angles, including carboxylase biotinylation,

metabolome, and transcriptome in healthy adults. The identification of sensitive and robust markers of biotin status would represent a major advance in our pursuit of the quantification of human biotin requirements. In this study, we devised a biotin feeding protocol to create states of biotin depletion, biotin sufficiency, and biotin supplementation in healthy women and men. We assessed the suitability of 20 distinct markers to identify those that reliably discriminate among the subjects in the three treatment groups. Only two markers met our stringent requirements and can be considered reliable markers of biotin status. Some of the other markers might be useful for use in future population studies, but only if used in combination with additional markers.

SUBJECTS AND METHODS

Study Principle

This is a randomized cross-over outpatient study. Defined states of biotin supply were established by supplementing the study subjects with drinks containing spray-dried egg white. Egg white contains the protein avidin, which binds biotin tightly and makes the vitamin unavailable for intestinal absorption[11,28]. Graded levels of chemically pure biotin were added back to the egg white shakes, thereby inducing states of biotin depletion, biotin sufficiency, and biotin supplementation (see below).

Study subjects

Seventeen apparently healthy adults (7 men and 10 women) aged 21 to 45 years completed this study. Exclusion criteria included a recent history of smoking[36], inborn errors of biotin metabolism (carboxylase deficiency, holocarboxylase synthetase deficiency, and biotinidase deficiency)[42,44], use of vitamin supplements, use of anticonvulsants[19,33], pregnancy[25,26], and lactation[24,30]. Women were tested for pregnancy prior to each period of egg white feeding by using a commercial pregnancy test. Subjects were allowed to engage in normal daily activities. After the end of the study, one male subject (21 years old) admitted that he did not comply with the study protocol and his data were excluded from analysis. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institutional Review Board of the University of Nebraska-Lincoln, USA. Subjects signed an informed consent prior to enrolling in the study.

Study protocol

The entire study took each subject 15 weeks to complete (Figure 2.1). During the first two weeks, subjects consumed a regular, self-selected diet without the use of any vitamin supplements. The objective of this adjustment period was to eliminate excess vitamins from inadvertent use of vitamin supplements before the study began. Subjects were instructed to avoid biotin-rich foods such as yeast, livers, meats, cereals, mayonnaise, and eggs[6], and were provided with a list of foods to avoid. After completion of the adjustment period, each subject completed the following three treatment phases (three weeks each)[40] in randomized order: biotin depletion (zero dietary biotin, representing levels seen in individuals consuming diets containing raw egg

white and individuals with undiagnosed deficiencies of biotinidase)[3,43], biotin sufficiency (30 µg/d, representing recommendations for Adequate Intake of biotin)[30], and biotin supplementation (600 µg/d, representing the level of biotin in typical over-the-counter supplement users)[18,45]. In the second week of each treatment, participants filled out 3-day dietary records in order to estimate biotin intake. Periods of biotin-defined diets were interrupted by two weeks with no biotin treatment. These periods are sufficiently long for biotin levels to return to baseline level[14,28,40]. Subjects consumed a regular, self-selected diet (see above), modified only by the amounts of biotin during treatment phases.

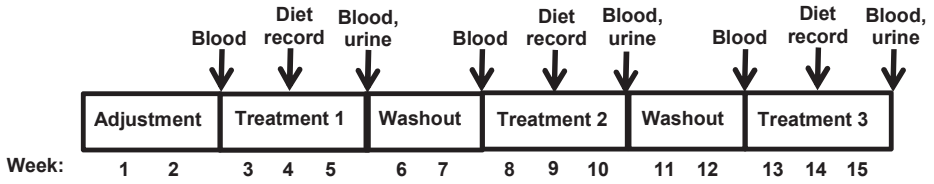

Figure 2.1. Study Protocol

During treatment, dietary biotin was adjusted by consuming an egg white drink (200 ml) with every major meal (breakfast, lunch, dinner) of the day. The amount of egg white in the drinks was chosen as follows. First, the amount of avidin in spray-dried egg white (The Barry Farm, Cridersville, OH) was titrated based on HABA (2-(4'hydroxyazobenzene)-benzoic acid) assay (*n* 8)[7,10]. Avidin activity (µg/ml dried egg white) was calculated as $(3.1 \times 68{,}000_{\text{extinction coefficient}} \times A_{3(A1\text{-}A2)} \times 1000)/(3 \times 34{,}000 \times 4) \times 5/0.2$ g dried egg white, based on the method of Green[7,10] where A_1 = optical density (OD) value of the supernatant of the egg white solution plus HABA at 500 nm, A_2 = OD at 500 nm after addition of biotin to the egg white HABA solution, and $A_3 = A_1$ minus A_2.

One gram of egg white contained approximately 600 μg of avidin. Second, we calculated the amount of egg white needed to bind the biotin in an average diet (30-70 μg/d)[30] including an allowance for microbial biotin synthesis and diets that were inadvertently high in biotin. Assuming that 1 μg of avidin binds 0.014 μg biotin[7], we prepared egg white drinks containing 7.1 mg of avidin in 200 ml (= one serving), which is sufficient to bind about 100 μg of biotin. Spray-dried egg white was dissolved in orange juice to improve palatability; the juices were stored at 4°C for up to two days before consumption. This drink was used for depleting subjects of biotin. For biotin-sufficient subjects, biotin was added back to the drinks to saturate all the avidin plus provide an additional 30 μg/d. For biotin-supplemented subjects, the amount of available biotin was adjusted to 600 μg/d.

Sample collection

Blood samples were collected after an overnight fast by an experienced phlebotomist at the University Health Center of Nebraska-Lincoln. One hundred-eighty milliliters of fasting blood was collected after the initial two-week adjustment period and on the last day of each treatment period. In addition, 30 ml of fasting blood was collected after the two-week washout period of each treatment. Lymphocytes were isolated by density gradient centrifugation and counted using a hemocytometer[47]. Aliquots of twenty million and two million cells were frozen (-80°C) for subsequent carboxylase analysis and for isolation of total RNA, respectively. Spot urines were collected after an overnight fast at the end of each treatment phase. Urines were subaliquoted to avoid repeated freeze-thaw cycles and frozen at -20°C for the subsequent analyses of organic acids, biotin, and creatinine.

Sample analysis

The abundance of biotinylated (holo-)carboxylases was quantified in lymphocyte extracts as described[14], using 20 µg of protein and 3-8 % Tris-Acetate gels (Invitrogen; Carlsbad, CA). Holocarboxylase-bound biotin in transblots was probed with fluorophore-labeled streptavidin; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as loading control[32]. The membrane was scanned using the 800CW channel in an infrared imaging system (Odyssey LI-COR, Lincoln, NE) and band intensities were quantified by gel densitometry[14].

Total RNA was extracted from lymphocytes using the ZR RNA MicroPrep (Zymo Research, Irvine, CA) following the manufacturer's instructions. RNA was reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The abundance of mRNA coding for the sodium-dependent multivitamin transporter (SMVT), monocarboxylate transporter 1 (MCT1), HLCS, ACC1, and MCC (beta-chain) were quantified by quantitative real-time PCR using Quanta Perfecta Power SYBR Green and the cycle threshold method[17,32]. The abundance of GAPDH mRNA was used to normalize data for efficiency of PCR reactions. PCR primers were the same as reported previously[14]. Our rationale for focusing on these five genes was that they play a role in biotin metabolism[50] and because their expression depended on biotin in previous studies in human cell cultures[14].

The concentrations of biotin in urine was quantified by avidin-binding assay as described[18]. Since urine was collected as spot urines (as opposed to 24-h collections), urinary biotin was normalized by the excretion of creatinine to account for possible

incomplete collections and dilution effects. Creatinine was quantified using the standard picric acid method of Jaffe[31]. Concentration of urinary organic acids was determined by a capillary electrophoresis method in our laboratory. Briefly, urine samples were thawed and centrifuged at 16100 ***g*** for one minute. One-hundred microliters of supernatant were lyophilized and weights of the freeze-dried samples were recorded for data normalization. After being re-dissolved in 50 µl of nanopure water, samples were injected into a 50 cm bare fused silica capillary (i.d. 50 µm) under the pressure of 0.5 psi for 5 s. Various organic acids were resolved at 15 kV and 25ºC using a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) equipped with 200 nm UV filter while 250 mM phosphate buffer (pH 6.0) with 0.1 mM hexadecyltrimethylammonium bromide and 5 % methanol (by volume). Authentic standards of organic acid were purchased from Sigma-Aldrich (St. Louis, MO). The following nine organic acids were quantified by a standard curve method and their urinary excretion was normalized by the excretion of creatinine: acetate, alpha-ketoglutarate, butyrate, citrate, fumarate, malate, oxalate, succinate and 3-Hydroxyisovaleric Acid. Our rationale for focusing on these organic acids was that they are implicated in biotin-dependent pathways in intermediary metabolism and that, for some of them, links have been established with biotin deficiency[20,50].

Statistical analysis

To test for homogeneity of variances, Barlett's test or Levene's test was performed[13]. Data was log transformed if variances were heterogeneous. The significance of differences among groups was tested with one-way ANOVA and Fisher's Protected Least Significant Difference procedure for posthoc testing[13]. StatView 5.0.1

(SAS Institute; Cary, NC) was used to perform all calculations. Differences are considered significant if $P<0.05$. Data are expressed as mean (SD).

RESULTS

Holo-carboxylases

Biotinylated holo-MCC (molecular mass = 83 kDa) and holo-PCC (molecular mass = 80 kDa) from lymphocyte extracts were easily detectable in streptavidin blots and appear to be reliable markers for biotin status (Figure 2.2). Note that streptavidin blotting detects the biotinylated alpha chains of these carboxylases, but not the non-biotinylated beta chains. While the abundance of holo-PC also appeared to respond to dietary biotin, the signal produced by holo-PC was faint and hard to detect, suggesting that PC might not be a good marker for biotin status. Holo-ACC1 and ACC2 were not detectable in streptavidin blots of lymphocyte extracts, which is consistent with previous studies in human lymphoid cell cultures[18]. Gels were equally loaded, judged by the abundance of the housekeeping protein GAPDH.

Gel densitometry analysis of holo-MCC and holo-PCC is consistent with the notion that these proteins are good markers for biotin status. The abundance of holo-MCC (arbitrary units) was 4.1 (SD 2.6) in biotin-depleted subjects, 8.2 (SD 3.5) in biotin-sufficient subjects, and 15.7 (SD 7.5) in biotin-supplemented subjects ($0.0001<P<0.0004$ for all possible comparisons, n 16). Likewise, the abundance of holo-PCC (arbitrary units) was 4.1 (SD 1.9) in biotin-depleted subjects, 9.1 (SD 3.3) in biotin-sufficient subjects, and 17.0 (SD 5.7) in biotin-supplemented subjects ($P<0.0001$ for all possible comparisons, n 16).

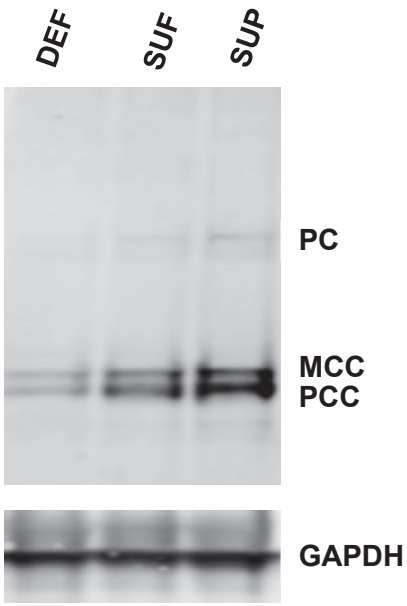

Figure 2.2. Abundance of biotinylated (holo) pyruvate carboxylase (PC), 3-methylcrotonyl-CoA carboxylase (MCC), and propionyl-CoA carboxylase (PCC) in lymphocytes from biotin-deficient (DEF), biotin-sufficient (SUF), and biotin-supplemented (SUP) healthy adults (top gel). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control (bottom gel). The image was cropped from an original full size scanned picture.

Real-Time PCR

The abundance of mRNA coding for the biotin transporters SMVT and MCT1, the biotin-dependent carboxylases ACC1 and MCC (beta-chain), and HLCS was not significantly different among treatment groups (Figure 2.3), contrary to previous observations in human cell cultures[14]. This apparent absence of effect can largely be explained by the large inter-individual variations among subjects. When men and women were analyzed separately, the results were the same as for the pooled data set (not shown). We conclude that the abundance of mRNA coding for genes in biotin metabolism is not a good marker of biotin status in outpatient studies.

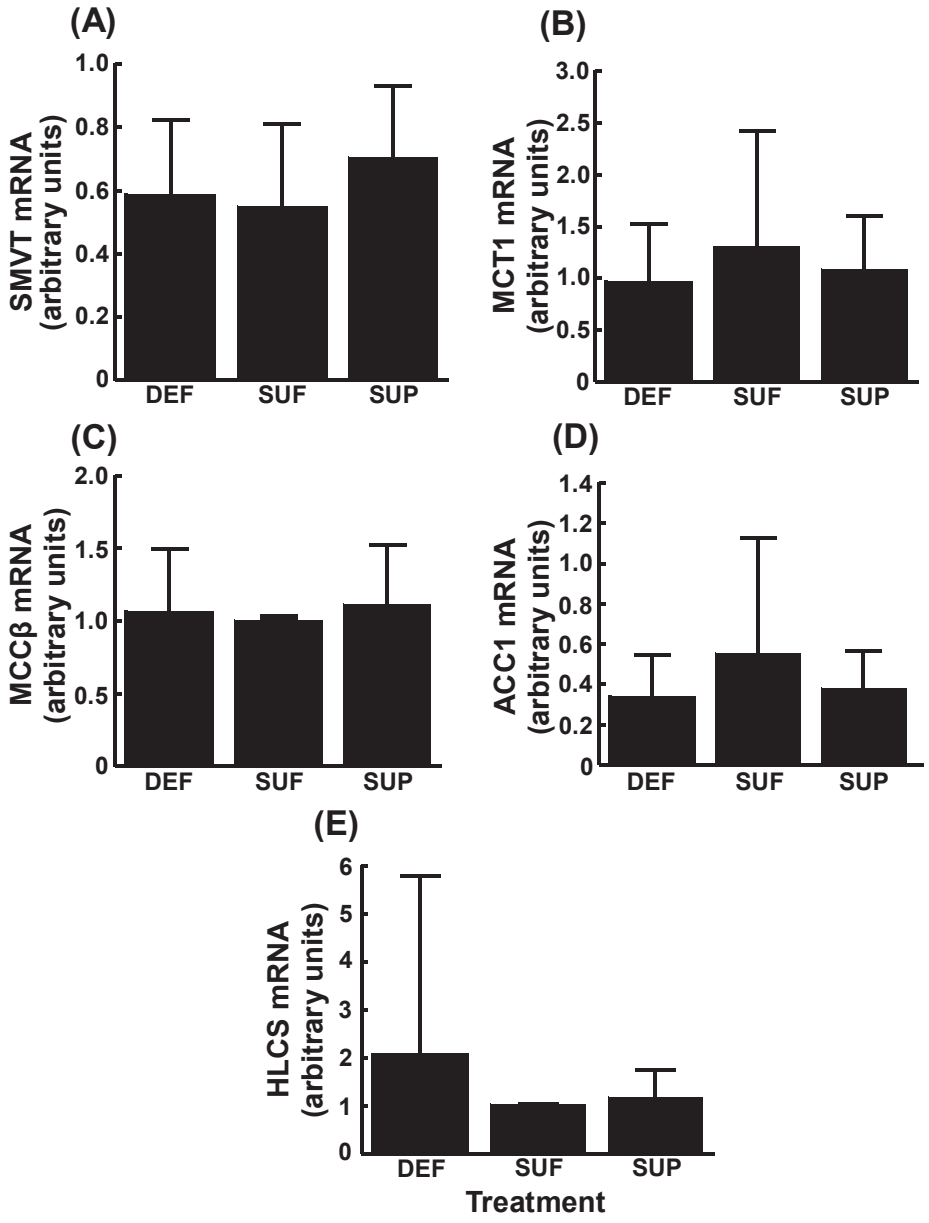

Figure 2.3. Abundance of mRNA coding for SMVT (A), MCT1 (B), MCCβ (C), ACC1 (D), and HLCS (E) in lymphocytes from biotin-deficient (DEF), biotin-sufficient (SUF), and biotin-supplemented (SUP) healthy adults. Values are means, with their standard deviations represented by vertical bars (*n* 16, 0.0784<*P*<0.9823).

Excretion of biotin in urine

The urinary excretion of biotin was up to five times higher in biotin-supplemented subjects compared with the other treatment groups (Table 2.1). There was a trend toward higher urinary biotin in biotin-sufficient individuals compared to biotin-depleted individuals, but the difference was not statistically significant. This apparent absence of effect can largely be explained by the large inter-individual variations among subjects. When men and women were analyzed separately, the results were the same as for the pooled data set (not shown). We conclude that the urinary excretion of biotin is a good marker for identifying supplement users, but is not a good marker to distinguish deficient from sufficient individuals in outpatient studies.

Table 2.1. Urinary excretion of biotin in three treatment groups (*n* 16). (Mean values and standard deviations)

	Urinary biotin (nmol biotin/μmol creatinine)	
Treatment	Mean	SD
Biotin deficient	0.52[a]	1.19
Biotin sufficient	0.74[a]	1.13
Biotin supplemented	2.54[b]	2.01

[a,b]Rows not sharing the same letter are significantly different ($0.0001<P<0.16$).

Excretion of organic acids in urine

The urinary excretion of 3-HIA was, on average, two times higher in biotin-deficient subjects compared with the other treatment groups (Figure 2.4A). The use of 3-HIA as a marker of biotin status is limited by the following characteristics. First, the excretion of 3-HIA does not distinguish biotin-sufficient and biotin-supplemented subjects. Second, for 8 of the 16 subjects the urinary excretion of 3-HIA did not increase during biotin depletion compared with other phases (Figure 2.4B) suggesting that the urinary excretion of 3-HIA has a tendency to produce false negatives in biotin-deficient subjects. For clarity, we show values only for those eight subjects for which urinary 3-HIA did not respond to dietary biotin; for comparison the figure also includes the data for one subject that showed the expected pattern of a higher excretion of 3-HIA during biotin depletion compared with other phases. When men and women were analyzed separately, the results were the same as for the pooled data set (not shown).

Two of the remaining organic acids produced potentially interesting trends regarding their urinary patterns, although effects did not reach a statistically significant level. Urinary citrate was positively linked with biotin intake (Figure 2.5A), while urinary malate was negatively linked with biotin intake (Figure 2.5B). Results were the same when men and women were analyzed separately. The urinary excretion of acetate, alpha-ketoglutarate, butyrate, fumarate, oxalate, and succinate did not depend on biotin intake.

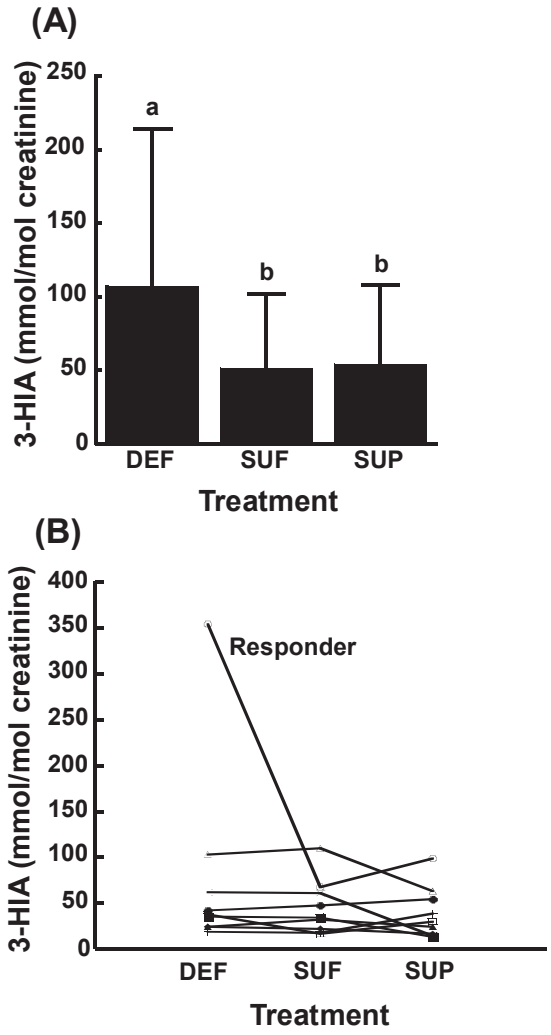

Figure 2.4. (A) Average urinary excretion of 3-HIA in biotin-deficient (DEF), biotin-sufficient (SUF), and biotin-supplemented (SUP) healthy adults. Values are means, with their standard deviations represented by vertical bars ($0.04<P<0.98$ for bars not sharing the same letter, n 16). (B) Individual patterns of the eight subjects in which urinary 3-HIA did not respond to dietary biotin, and one of the subjects that exhibited the expected pattern of high urinary 3-HIA during biotin depletion (denoted "Responder").

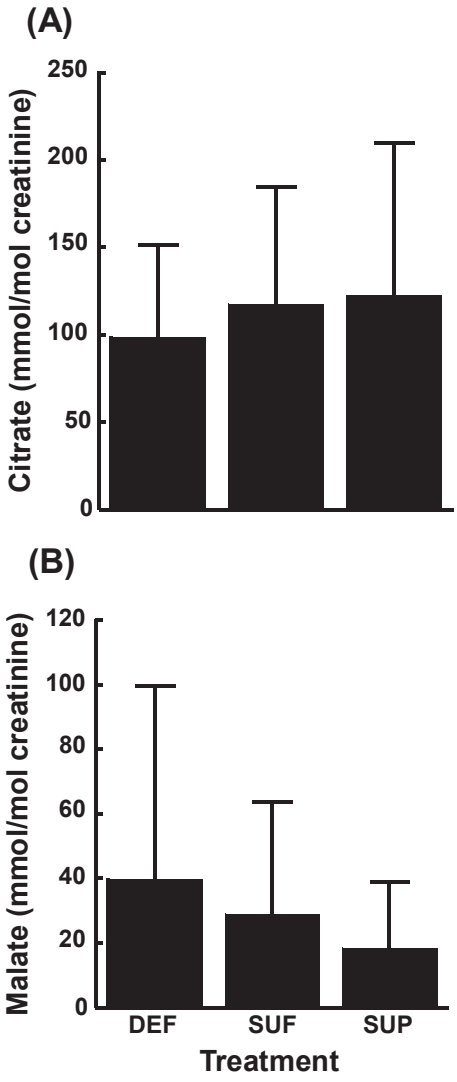

Figure 2.5. Urinary excretion of citrate (A) and malate (B) in biotin-deficient (DEF), biotin-sufficient (SUF), and biotin-supplemented (SUP) healthy adults. Values are means, with their standard deviations represented by vertical bars ($0.4620<P<0.6943$, n 16).

Dietary records

The dietary intake of biotin was of the expected magnitude and well within the amount that can be bound by the avidin in the egg white drinks. The intake of biotin over a 3-d period was 37.9 μg (SD 23.5) in biotin-depleted subjects, 65.2 μg (SD 71.4) in biotin-sufficient subjects, and 49.3 μg (SD 29.2) in biotin-supplemented subjects (*n* 16), excluding the biotin that was added back to drinks. These values should be considered crude estimates because there is currently no database that accurately quantifies biotin in foods[9,38]. Despite the limited accuracy of existing databases, the intake estimates reported here are consistent with the notion that subjects avoided foods rich in biotin throughout the study.

Efficacy of washout

Adjustment and washout periods were chosen sufficiently long to allow for biotin to baseline levels. When carboxylase-bound biotin in lymphocyte extracts was probed with streptavidin, no apparent difference among the various phases was detected for any of the study subjects. Figure 2.6 depicts the raw data from one subject as a representative example. The abundance of GAPDH was used as loading control.

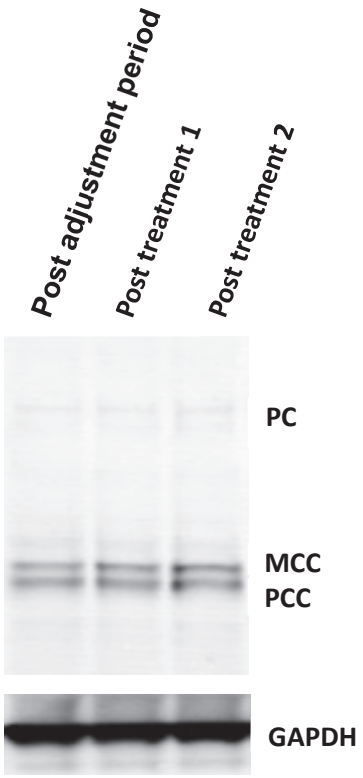

Figure 2.6. The abundance of biotinylated (holo) pyruvate carboxylase (PC), 3-methylcrotonyl-CoA carboxylase (MCC), and propionyl-CoA carboxylase (PCC) in lymphocytes from biotin-deficient (DEF), biotin-sufficient (SUF), and biotin-supplemented (SUP) subjects at the end of adjustment and washout phases (top gel). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control (bottom gel). The image was cropped from an original full size scanned picture.

DISCUSSION

This paper is the first study that uses an outpatient feeding protocol in normal healthy adults to assess the effects of deficiency, sufficiency, and supplementation on a comprehensive set of 20 variables from proteomics, transcriptomics, and metabolomics. The study provides compelling evidence that the abundance of biotinylated (holo) MCC and PCC in lymphocytes are the only markers that can reliably discriminate among the three states of biotin intake that were tested. This observation is consistent with previous studies in human lymphoid cell cultures, which also suggest that biotinylation of MCC and PCC is responsive to biotin intake[14,18]. Previous studies in healthy adults[21] and rats[23] also led to the conclusion that the activity of PCC is a good marker of biotin status in the population. Those studies used the incorporation of radiolabeled bicarbonate for assessing carboxylase activity while in our study we quantified the binding of biotin to carboxylases. In previous studies we have demonstrated that both carboxylase activity and carboxylase biotinylation correlate well[18]. However, we believe that the dependence on radiolabeled compounds is a meaningful limitation when using carboxylases as markers for biotin status. We also believe that streptavidin blots of carboxylases has the additional advantage of assessing multiple carboxylases in one single run, while activity assays require distinct analytical procedures for each of these enzymes[49].

When biotin is insufficient during leucine metabolism, it shunts the pathway to an alternative pathway leading to the formation of 3-HIA[50]. Urinary 3-HIA is generally considered a useful marker for biotin status[20,29,41]. Based on the findings in this study, the usefulness of 3-HIA as a marker of biotin status needs to be re-evaluated because of the following concerns. First, urinary 3-HIA does not permit discriminating biotin-

sufficient and biotin-supplemented individuals. Second, the average urinary excretion of 3-hydroxyisovaleric acid was greater in biotin-deficient subjects compared with other treatment groups, but produced a meaningful number of false negative results when looking at the individuals in the biotin-deficient group. More recently, carnitine conjugates of 3-HIA have been recommended as markers of biotin status[12,27,41]. Carnitine conjugates of 3-HIA were not tested in this study, but might be a useful alternative to free 3-HIA. When validating carnitine conjugates in future studies, great care should be taken (a) to document inter-individual variation, (b) to test for false negatives in a population of biotin-depleted individuals, and (c) to determine whether these conjugates discriminate between sufficient and supplemented individuals. Analysis of urinary citrate and malate might be a useful alternative or addition to the analysis of 3-HIA and its carnitine conjugate. Our study suggests that urinary citrate and malate are positively and negatively linked with biotin intake, despite the effects not reaching statistical significance in a sample size as small as 16 subjects. However, the combined use of three to four organic acids for assessing biotin status can be achieved within one single analytical run, and might permit the identification of supplement users (high citrate, low 3-HIA, low malate) while minimizing the risk of false negatives in biotin-deficient individuals (low citrate, high 3-HIA, high malate).

One could consider using urinary biotin as a marker of biotin status. Our studies suggest that the urinary excretion of biotin reliably identifies biotin-supplemented subjects, but does not distinguish between biotin-depleted and biotin-sufficient individuals. Also, urine contains biotin metabolites such as bisnorbiotin, biotin-*d,l*-sulfoxides, biotin sulfone, bisnorbiotin methyl ketone, and tetranorbiotin-*l*-sulfoxide[22,46].

Typical biotin assays do not quantify these metabolites accurately, and their analysis is reserved for specialty laboratories.

Of the 20 potential markers of biotin status that were tested in this study, quantification of mRNA abundance returned the least compelling results. None of the five genes from biotin turnover was linked in any way to biotin intake. This can be attributed to large inter-individual variations in the expression of these genes. This observation is in striking contrast to studies in cell cultures and laboratory animals where the expression of these genes depended on biotin[14,35,37]. We propose that the genetic diversity in humans and environmental factors create a unique scenario and those studies in cell cultures and inbred animals need to be interpreted with care.

Collectively, we conclude that the number of reliable markers of biotin status is limited and that more groundwork needs to be done before human biotin requirements can be quantified with a reasonable level of certainty.

ACKNOWLEDGEMENTS

The present study was a contribution of the University of Nebraska Agricultural Research Division, supported in part by funds provided through the Hatch Act. Additional support was provided by National Institutes of Health grants (Grant nos. DK063945, DK077816, and DK082476). The contributions of authors were as follow: J. Z., V. L. S., D. W. and D. G. designed the research; D. G. conducted the preliminary research; D. G. and W. K. E. conducted the research; B. H. L. conducted the Capillary Electrophoresis analyses; J. Z., D. W., D. G. and W. K. E. analyzed the data; W. K. E. and J. Z. prepared the manuscript. All authors read, edited and approved the final manuscript. The authors declare that there is no conflict of interests.

REFERENCES

1. Bailey LM, Ivanov RA, Wallace JC *et al.* (2008) Artifactual detection of biotin on histones by streptavidin. *Anal Biochem* **373**, 71-77.
2. Bao B, Pestinger V, I. HY *et al.* (2011) Holocarboxylase synthetase is a chromatin protein and interacts directly with histone H3 to mediate biotinylation of K9 and K18. *J Nutr Biochem* **22**, 470-475.
3. Baugh CM, Malone JH & Butterworth CE, Jr. (1968) Human biotin deficiency: a case history of biotin deficiency induced by raw egg consumption in a cirrhotic patient. *Am J Clin Nutr* **21**, 173-182.
4. Camporeale G, Oommen AM, Griffin JB *et al.* (2007) K12-biotinylated histone H4 marks heterochromatin in human lymphoblastoma cells. *J Nutr Biochem* **18**, 760-768.
5. Camporeale G, Shubert EE, Sarath G *et al.* (2004) K8 and K12 are biotinylated in human histone H4. *Eur J Biochem* **271**, 2257-2263.
6. Camporeale G & Zempleni J (2006) Biotin. In *Present Knowledge in Nutrition*, 9th ed., pp. 314-326 [BA Bowman and RM Russell, editors]. Washington, D.C.: International Life Sciences Institute.
7. Durance TD (1991) Residual Avidin Activity in Cooked Egg White Assayed with Improved Sensitivity. *J Food Sci* **56**, 707-709.
8. Filenko NA, Kolar C, West JT *et al.* (2011) The role of histone H4 biotinylation in the structure and dynamics of nucleosomes. *PLoS ONE* **6**, e16299.
9. Food Standard Australia New Zealand (2010) NUTTAB 2010 Online Searchable Database.
http://www.foodstandards.gov.au/consumerinformation/nuttab2010/nuttab2010onlinesearchabledatabase/onlineversion.cfm?&action=search (accessed June 25 2012).
10. Green NM (1965) A spectrophotometric assay for avidin and biotin based on binding of dyes by avidin. *Biochem J* **94**, 23c-24c.
11. Green NM (1975) Avidin. *Adv Protein Chem* **29**, 85-133.
12. Horvath TD, Stratton SL, Bogusiewicz A *et al.* (2010) Quantitative measurement of urinary excretion of 3-hydroxyisovaleryl carnitine by LC-MS/MS as an indicator of biotin status in humans *Anal Chem* **82**, 9543-9548.
13. SAS Institute (1999) *StatView Reference*. 3rd ed. Cary, NC: SAS Publishing.
14. Kaur Mall G, Chew YC & Zempleni J (2010) Biotin requirements are lower in human Jurkat lymphoid cells but homeostatic mechanisms are similar to those of HepG2 liver cells. *J Nutr* **140**, 1086-1092.
15. Kobza K, Camporeale G, Rueckert B *et al.* (2005) K4, K9, and K18 in human histone H3 are targets for biotinylation by biotinidase. *FEBS J* **272**, 4249-4259.
16. Kuroishi T, Rios-Avila L, Pestinger V *et al.* (2011) Biotinylation is a natural, albeit rare, modification of human histones. *Mol Genet Metab* **104**, 537-545.
17. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408.
18. Manthey KC, Griffin JB & Zempleni J (2002) Biotin supply affects expression of biotin transporters, biotinylation of carboxylases, and metabolism of interleukin-2 in Jurkat cells. *J Nutr* **132**, 887-892.

19. Mock DM & Dyken ME (1997) Biotin catabolism is accelerated in adults receiving long-term therapy with anticonvulsants. *Neurology* **49**, 1444-1447.
20. Mock DM, Henrich CL, Carnell N *et al.* (2002) Indicators of marginal biotin deficiency and repletion in humans: validation of 3-hydroxyisovaleric acid excretion and a leucine challenge. *Am J Clin Nutr* **76**, 1061-1068.
21. Mock DM, Henrich CL, Carnell N *et al.* (2002) Lymphocyte propionyl-CoA carboxylase and accumulation of odd-chain fatty acid in plasma and erythrocytes are useful indicators of marginal biotin deficiency. *J Nutr Biochem* **13**, 462-470.
22. Mock DM, Lankford GL & Cazin Jr J (1993) Biotin and biotin analogs in human urine: biotin accounts for only half of the total. *J Nutr* **123**, 1844-1851.
23. Mock DM & Mock NI (2002) Lymphocyte propionyl-CoA carboxylase is an early and sensitive indicator of biotin deficiency in rats, but urinary excretion of 3-hydroxypropionic acid is not. *J Nutr* **132**, 1945-1950.
24. Mock DM, Mock NI & Dankle JA (1992) Secretory patterns of biotin in human milk. *J Nutr* **122**, 546-552.
25. Mock DM, Stadler D, Stratton S *et al.* (1997) Biotin status assessed longitudinally in pregnant women. *J Nutr* **127**, 710-716.
26. Mock DM & Stadler DD (1997) Conflicting indicators of biotin status from a cross-sectional study of normal pregnancy. *J Am Coll Nutr* **16**, 252-257.
27. Mock DM, Stratton SL, Horvath TD *et al.* (2011) Urinary excretion of 3-hydroxyisovaleric acid and 3-hydroxyisovaleryl carnitine increases in response to a leucine challenge in marginally biotin-deficient humans. *J Nutr* **141**, 1925-1930.
28. Mock N, Malik M, Stumbo P *et al.* (1997) Increased urinary excretion of 3-hydroxyisovaleric acid and decreased urinary excretion of biotin are sensitive early indicators of decreased status in experimental biotin deficiency. *Am J Clin Nutr* **65**, 951-958.
29. Mock NI, Mock DM, Malik M *et al.* (1995) Urinary excretion of biotin and 3-hydroxyisovaleric acid (3-HIA) are early indicators of biotin deficiency. *FASEB J* **9**, A985 [abstract].
30. National Research Council (1998) *Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline*. Washington, DC: National Academy Press.
31. O'Brien D, Ibbott FA & Rodgerson DO (1968) *Laboratory manual of pediatric micro-biochemical techniques*. 4th ed. New York: Hoeber Medical Division, Harper & Row, Publishers, Inc.
32. Pestinger V, Wijeratne SSK, Rodriguez-Melendez R *et al.* (2011) Novel histone biotinylation marks are enriched in repeat regions and participate in repression of transcriptionally competent genes. *J Nutr Biochem* **22**, 328-333.
33. Rathman SC, Eisenschenk S & McMahon RJ (2002) The Abundance and Function of Biotin-Dependent Enzymes Are Reduced in Rats Chronically Administered Carbamazepine. *J Nutr* **132**, 3405-3410
34. Rios-Avila L, Pestinger V, Wijeratne SSK *et al.* (2012) K16-biotinylated histone H4 is overrepresented in repeat regions and participates in the repression of transcriptionally competent genes in human Jurkat lymphoid cells. *J Nutr Biochem* (in press).

35. Rodriguez-Melendez R, Cano S, Mendez ST *et al.* (2001) Biotin regulates the genetic expression of holocarboxylase synthetase and mitochondrial carboxylases in rats. *J Nutr* **131**, 1909-1913.
36. Sealey WM, Teague AM, Stratton SL *et al.* (2004) Smoking accelerates biotin catabolism in women. *Am J Clin Nutr* **80**, 932-935.
37. Solorzano-Vargas RS, Pacheco-Alvarez D & Leon-Del-Rio A (2002) Holocarboxylase synthetase is an obligate participant in biotin-mediated regulation of its own expression and of biotin-dependent carboxylases mRNA levels in human cells. *Proc Natl Acad Sci USA* **99**, 5325-5330.
38. Staggs CG, Sealey WM, McCabe BJ *et al.* (2004) Determination of the biotin content of select foods using accurate and sensitive HPLC/avidin binding. *J Food Compost Anal* **17**, 767-776.
39. Stanley JS, Griffin JB & Zempleni J (2001) Biotinylation of histones in human cells: effects of cell proliferation. *Eur J Biochem* **268**, 5424-5429.
40. Stratton SL, Henrich CL, Matthews NI *et al.* (2012) Marginal Biotin Deficiency Can Be Induced Experimentally in Humans Using a Cost-Effective Outpatient Design. *J Nutr* **142**, 22-26.
41. Stratton SL, Horvath TD, Bogusiewicz A *et al.* (2011) Urinary excretion of 3-hydroxyisovaleryl carnitine is an early and sensitive indicator of marginal biotin deficiency in humans. *J Nutr* **141**, 353-358.
42. Weiner DL, Grier RE & Wolf B (1985) A Bioassay for determining biotinidase activity and for discriminating biocytin from biotin using holocarboxylase synthetase-deficient cultured fibroblasts. *J Inherit Metab Dis* **8**, 101-102.
43. Wolf B & Heard GS (1991) Biotinidase deficiency. In *Advances in Pediatrics*, pp. 1-21 [L Barness and F Oski, editors]. Chicago, IL: Medical Book Publishers.
44. Wolf B, Heard GS, Weissbecker KA *et al.* (1985) Biotinidase deficiency: initial clinical features and rapid diagnosis. *Ann Neurol* **18**, 614-617.
45. Zempleni J, Helm RM & Mock DM (2001) In vivo biotin supplementation at a pharmacologic dose decreases proliferation rates of human peripheral blood mononuclear cells and cytokine release. *J Nutr* **131**, 1479-1484.
46. Zempleni J, McCormick DB & Mock DM (1997) Identification of biotin sulfone, bisnorbiotin methyl ketone, and tetranorbiotin-*l*- sulfoxide in human urine. *Am J Clin Nutr* **65**, 508-511.
47. Zempleni J & Mock DM (1998) Uptake and metabolism of biotin by human peripheral blood mononuclear cells. *Am J Physiol Cell Physiol* **275**, C382-C388.
48. Zempleni J & Mock DM (2000) Biotin. In *Modern Analytical Methodologies on Fat and Water-Soluble Vitamins*, pp. 389-409 [WO Song and GR Beecher, editors]. New York, NY: Wiley & Sons, Inc.
49. Zempleni J, Trusty TA & Mock DM (1997) Lipoic acid reduces the activities of biotin-dependent carboxylases in rat liver. *J Nutr* **127**, 1776-1781.
50. Zempleni J, Wijeratne SS & Hassan YI (2009) Biotin. *Biofactors* **35**, 36-46.

APPENDIX

1. (A) Data for MCC gel densitometry (arbitrary units)

D	1	2.08	SUF	1	4.73	SUP	1	19.26
D	2	4.31	SUF	2	8.52	SUP	2	11.45
D	3	2.1	SUF	3	4.63	SUP	3	9.02
D	4	1.95	SUF	4	5.54	SUP	4	15.64
D	5	2.81	SUF	5	8.39	SUP	5	13.02
D	6	1.36	SUF	6	9.06	SUP	6	13.16
D	7	2.44	SUF	7	7.17	SUP	7	9.44
D	8	2.48	SUF	8	9.64	SUP	8	11.61
D	9	4.84	SUF	9	11.31	SUP	9	17.54
D	10	11.06	SUF	10	16.66	SUP	10	25.8
D	11	3.83	SUF	11	5.59	SUP	11	7.83
D	12	3.87	SUF	12	10.43	SUP	12	13.43
D	13	7.42	SUF	13	13.07	SUP	13	20.03
D	14	3.1	SUF	14	3.03	SUP	14	10.03
D	15	7.06	SUF	15	7.17	SUP	15	15.75
D	16	4.4	SUF	16	6.38	SUP	16	37.56
Mean :		**4.07**			**8.21**			**15.66**
Standard Deviation :		**2.55**			**3.5**			**7.51**

(B) Data for PCC gel densitometry

D	1	1.44	SUF	1	5.66	SUP	1	13.46
D	2	6.61	SUF	2	10.16	SUP	2	15.87
D	3	2.43	SUF	3	6.94	SUP	3	12.46
D	4	2.06	SUF	4	7.5	SUP	4	17.11
D	5	3.16	SUF	5	10.78	SUP	5	18.26
D	6	3.14	SUF	6	14.16	SUP	6	28.14
D	7	2.81	SUF	7	11.57	SUP	7	13.83
D	8	6.5	SUF	8	14.97	SUP	8	20.6
D	9	3.74	SUF	9	9.43	SUP	9	15.7
D	10	8.2	SUF	10	13.55	SUP	10	23.19
D	11	4.32	SUF	11	6.86	SUP	11	12.36
D	12	3.03	SUF	12	8.47	SUP	12	12.29
D	13	4.2	SUF	13	6.96	SUP	13	13.61
D	14	3.08	SUF	14	3.13	SUP	14	10.11
D	15	6.1	SUF	15	6.76	SUP	15	14.75
D	16	4.44	SUF	16	8.15	SUP	16	29.8
Mean :		**4.08**			**9.07**			**16.97**
Standard Deviation:		**1.88**			**3.28**			**5.74**

2. (A) Data for Real-Time PCR: HLCS (arbitrary units)

D	2.41	SUF	1.007	SUP	0.687
D	1.012	SUF	1.002	SUP	1.099
D	0.743	SUF	1.035	SUP	0.913
D	1.213	SUF	1.015	SUP	0.714
D	15.667	SUF	1.015	SUP	1.101
D	0.902	SUF	1.047	SUP	1.704
D	0.383	SUF	1.015	SUP	0.602
D	3.356	SUF	1.004	SUP	2.077
D	0.877	SUF	1.001	SUP	1.183
D	1.054	SUF	1.027	SUP	1.263
D	0.603	SUF	1.004	SUP	0.398
D	0.566	SUF	1.028	SUP	0.725
D	0.391	SUF	1.009	SUP	0.592
D	1.626	SUF	1.032	SUP	1.853
D	1.062	SUF	1.002	SUP	2.104
D	1.487	SUF	1.079	SUP	1.669
Mean :	**2.08**		**1.02**		**1.17**
Standard Deviation :	**3.7**		**0.02**		**0.56**

(B) MCCβ

D	2.134	SUF	1.006	SUP	1.294
D	0.777	SUF	1.016	SUP	0.879
D	1.109	SUF	1.014	SUP	1.18
D	1.351	SUF	1.001	SUP	1.054
D	0.575	SUF	1.002	SUP	1.038
D	1.16	SUF	1.007	SUP	1.027
D	0.763	SUF	1.001	SUP	0.692
D	0.955	SUF	1.017	SUP	1.278
D	0.955	SUF	1.012	SUP	0.988
D	1.454	SUF	1.004	SUP	1.483
D	0.724	SUF	1.022	SUP	0.648
D	0.608	SUF	1.001	SUP	0.732
D	0.676	SUF	1.004	SUP	0.893
D	1.708	SUF	1.002	SUP	1.914
D	0.878	SUF	1.002	SUP	0.751
D	1.206	SUF	1.004	SUP	2.007
Mean :	**1.06**		**1.01**		**1.12**
Standard Deviation :	**0.43**		**0.01**		**0.4**

(C) ACCα:

	D	0.746	SUF	0.406	SUP	0.783
	D	0.244	SUF	0.256	SUP	0.526
	D	0.269	SUF	0.315	SUP	0.416
	D	0.617	SUF	0.727	SUP	0.324
	D	0.275	SUF	0.208	SUP	0.301
	D	0.223	SUF	0.5	SUP	0.31
	D	0.095	SUF	0.346	SUP	0.356
	D	0.196	SUF	0.218	SUP	0.274
	D	0.735	SUF	1.484	SUP	0.646
	D	0.22	SUF	0.758	SUP	0.213
	D	0.338	SUF	0.467	SUP	0.182
	D	0.145	SUF	0.208	SUP	0.161
	D	0.198	SUF	0.239	SUP	0.141
	D	0.362	SUF	0.195	SUP	0.346
	D	0.347	SUF	0.252	SUP	0.501
	D	0.473	SUF	2.314	SUP	0.616
Mean :		**0.34**		**0.56**		**0.38**
Standard Deviation :		**0.2**		**0.57**		**0.19**

(D) SMVT

	D	0.93	SUF	0.501	SUP	0.744
	D	0.422	SUF	0.346	SUP	0.717
	D	0.541	SUF	0.351	SUP	0.695
	D	0.71	SUF	0.536	SUP	0.721
	D	0.458	SUF	0.447	SUP	0.725
	D	0.517	SUF	0.603	SUP	0.602
	D	0.306	SUF	0.512	SUP	0.575
	D	0.472	SUF	0.379	SUP	0.702
	D	1.108	SUF	1.353	SUP	1.049
	D	0.578	SUF	0.694	SUP	0.694
	D	0.479	SUF	0.606	SUP	0.408
	D	0.284	SUF	0.323	SUP	0.362
	D	0.394	SUF	0.397	SUP	0.408
	D	0.892	SUF	0.574	SUP	1.17
	D	0.515	SUF	0.346	SUP	0.665
	D	0.784	SUF	0.851	SUP	1.028
Mean :		**0.59**		**0.55**		**0.7**
Standard Deviation :		**0.23**		**0.26**		**0.23**

(E) MCT1

	D	1.292	SUF	0.554	SUP	1.576
	D	0.575	SUF	0.5	SUP	1.322
	D	0.661	SUF	0.468	SUP	0.83
	D	1.151	SUF	1.224	SUP	0.849
	D	1.204	SUF	0.661	SUP	1.001
	D	0.692	SUF	1.791	SUP	0.875
	D	0.478	SUF	0.892	SUP	1.285
	D	0.502	SUF	0.602	SUP	0.763
	D	2.631	SUF	4.136	SUP	1.848
	D	0.871	SUF	2.899	SUP	0.692
	D	1	SUF	1.237	SUP	0.481
	D	0.416	SUF	0.47	SUP	0.372
	D	0.741	SUF	0.691	SUP	0.514
	D	1.561	SUF	0.891	SUP	2.116
	D	0.785	SUF	0.416	SUP	1.208
	D	1.099	SUF	3.181	SUP	1.628
Mean :		**0.98**		**1.29**		**1.09**
Standard Deviation :		**0.55**		**1.14**		**0.51**

3. Data for urinary excretion of organic acids (mmol/mol creatinine)
(A) 3-Hydroxyisovaleric acids (3-HIA)

D	1	355.018	SUF	1	67.794	SUP	1	98.894
D	2	42.633	SUF	2	47.822	SUP	2	54.401
D	3	103.095	SUF	3	109.841	SUP	3	63.674
D	4	88.602	SUF	4	18.856	SUP	4	62.939
D	5	24.913	SUF	5	32.056	SUP	5	23.948
D	6	38.117	SUF	6	16.331	SUP	6	30.592
D	7	35.094	SUF	7	34.372	SUP	7	13.907
D	8	175.982	SUF	8	72.329	SUP	8	100.321
D	9	331.372	SUF	9	144.809	SUP	9	181.149
D	10	79.571	SUF	10	56.403	SUP	10	33.03
D	11	61.971	SUF	11	60.998	SUP	11	14.11
D	12	89.645	SUF	12	31.891	SUP	12	32.651
D	13	158.006	SUF	13	49.232	SUP	13	61.492
D	14	19.081	SUF	14	17.892	SUP	14	39.022
D	15	24.519	SUF	15	22.174	SUP	15	16.176
D	16	86.09	SUF	16	35.833	SUP	16	35.989
Mean :		**107.11**			**51.16**			**53.89**
Standard Deviation :		**102.77**			**35.15**			**43.27**

(B) Citrate

D	1	212.964	SUF	1	212.663	SUP	1	241.654
D	2	89.024	SUF	2	87.049	SUP	2	268.207
D	3	112.282	SUF	3	122.588	SUP	3	155.229
D	4	84.096	SUF	4	84.75	SUP	4	72.056
D	5	35.98	SUF	5	80.776	SUP	5	99.592
D	6	31.387	SUF	6	53.271	SUP	6	32.268
D	7	48.463	SUF	7	61.505	SUP	7	54.365
D	8	124.035	SUF	8	134.676	SUP	8	109.61
D	9	177.971	SUF	9	291.417	SUP	9	311.668
D	10	59.421	SUF	10	126.627	SUP	10	69.139
D	11	95.026	SUF	11	87.342	SUP	11	36.263
D	12	154.28	SUF	12	187.627	SUP	12	179.259
D	13	111.207	SUF	13	164.254	SUP	13	148.116
D	14	56.228	SUF	14	66.995	SUP	14	80.198
D	15	137.295	SUF	15	74.239	SUP	15	56.403
D	16	53.182	SUF	16	44.203	SUP	16	37.833
Mean :		**98.93**			**117.5**			**121.99**
Standard Deviation :		**52.68**			**67.4**			**87.86**

(C) Malate

D	1	15.513	SUF	1	58.974	SUP	1	14.15
D	2	13.391	SUF	2	15.311	SUP	2	26.948
D	3	7.692	SUF	3	23.612	SUP	3	1.545
D	4	25.836	SUF	4	3.038	SUP	4	2.657
D	5	2.829	SUF	5	6.395	SUP	5	13.064
D	6	10.651	SUF	6	7.481	SUP	6	2.713
D	7	59.044	SUF	7	33.541	SUP	7	6.488
D	8	31.215	SUF	8	8.854	SUP	8	7.433
D	9	240.804	SUF	9	18.083	SUP	9	10.585
D	10	16.216	SUF	10	69.346	SUP	10	10.879
D	11	21.993	SUF	11	21.422	SUP	11	8.586
D	12	54.753	SUF	12	9.649	SUP	12	33.079
D	13	107.021	SUF	13	136.644	SUP	13	80.479
D	14	7.846	SUF	14	34.893	SUP	14	47.775
D	15	1.842	SUF	15	1.342	SUP	15	9.476
D	16	20.353	SUF	16	16.438	SUP	16	17.471
Mean :		**39.81**			**29.06**			**18.33**
Standard Deviation :		**60.02**			**34.56**			**20.63**

4. Individual line graph for urinary excretion of 3-HIA (mmol/mol creatinine)

Subjects	Deficient	Sufficient	Supplemented
S1	355.018	67.794	98.894
S2	42.633	47.822	54.401
S3	103.095	109.841	63.674
S5	24.913	32.056	23.948
S6	38.117	16.331	30.592
S7	35.094	34.372	13.907
S11	61.971	60.998	14.11
S14	19.081	17.892	39.022
S15	24.519	22.174	16.176

5. Urinary excretion of biotin (nmol biotin/µmol creatinine)

D	1	0.12	SUF	1	0.11	SUP	1	3.35
D	2	0.12	SUF	2	0.20	SUP	2	4.12
D	3	0.48	SUF	3	0.80	SUP	3	5.67
D	4	0.23	SUF	4	0.71	SUP	4	7.56
D	5	0.06	SUF	5	0.11	SUP	5	2.79
D	6	0.01	SUF	6	0.54	SUP	6	1.28
D	7	0.03	SUF	7	0.24	SUP	7	0.06
D	8	0.53	SUF	8	2.07	SUP	8	0.56
D	9	1.51	SUF	9	2.04	SUP	9	1.25
D	10	0.02	SUF	10	0.05	SUP	10	1.83
D	11	0.25	SUF	11	0.05	SUP	11	1.54
D	12	0.07	SUF	12	0.06	SUP	12	1.57
D	13	0.02	SUF	13	4.19	SUP	13	2.94
D	14	0.02	SUF	14	0.01	SUP	14	3.39
D	15	4.77	SUF	15	0.50	SUP	15	0.11
D	16	0.09	SUF	16	0.18	SUP	16	2.69
Mean :		**0.52**			**0.74**			**2.54**
Standard Deviation :		**1.19**			**1.13**			**2.01**