EFFECT OF BIOTIN SUPPLEMENTATION ON THE METABOLISM OF LACTATING DAIRY COWS

Dissertation

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By

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ABSTRACT

Biotin requirements for lactating dairy cows have not been established. Several studies observed that biotin supplementation increases milk yield by dairy cows, although this response was not consistent. The overall objective of this research was to enhance our understanding of when and how biotin supplementation increases milk production by lactating dairy cows. In the first study, we evaluated the urinary excretion of 3-hydroxy-isovaleric acid (3HIA) as a determinant of biotin status. We hypothesized that high-producing cows have a greater need for biotin, and therefore would excrete more 3HIA than low-producing cows. We also hypothesized that biotin supplementation decreases 3HIA excretion. Biotin supplementation increased milk yield in high-producing cows, but not in low-producing cows. Neither production nor biotin supplementation affected the basal urinary excretion of 3HIA. We concluded that urinary excretion of 3HIA is not a sensitive determinant of biotin status in lactating dairy cows. Other measures of biotin status, such as the concentration of avidin-binding substances (ABS) in plasma, milk and urine, were also evaluated. Contrary to our expectations, high-producing and low-producing cows consuming the control diet had similar ABS concentrations in plasma and milk and similar ABS to creatinine ratios in urine. A sensitive indicator of biotin status
for lactating dairy cows is still needed. The mechanism by which biotin supplementation increases milk production is not known. In the second study, we hypothesized that biotin supplementation increases the activity and the gene expression of propionyl-CoA carboxylase (PCC) and pyruvate carboxylase (PC) in the liver of high producing dairy cows. Biotin supplementation tended to increase the activity of PC, but did not affect the activity of PCC. The differential response to biotin supplementation suggests that PCC may have a higher priority for biotin than PC. Biotin supplementation did not affect the gene expression of PCC and PC. We concluded that biotin supplementation can affect the activity of biotin-dependent carboxylases in the liver of lactating dairy cows. Whether biotin supplementation increases glucose production in the liver still needs to be evaluated.
Dedicated to my beautiful wife Paula, my greatest finding at OSU,

and to Trinidad, our greatest creation in Ohio.
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Thanks God for all you have given to me.
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Discipline: Dairy Nutrition
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<tr>
<td>3HIA</td>
<td>3-hydroxy-isovaleric acid</td>
</tr>
<tr>
<td>ABS</td>
<td>Avidin-binding substances</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DAPA</td>
<td>7,8-diamino pelargonic acid</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>ECM</td>
<td>Energy-corrected milk</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas-liquid chromatography</td>
</tr>
<tr>
<td>HCS</td>
<td>Holocarboxylase synthetase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IVA</td>
<td>Isovaleric acid</td>
</tr>
<tr>
<td>KAPA</td>
<td>7-keto-8-amino pelargonic acid</td>
</tr>
<tr>
<td>MCC</td>
<td>3-methylcrotonyl-CoA carboxylase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PC</td>
<td>Pyruvate carboxylase</td>
</tr>
<tr>
<td>PCC</td>
<td>Propionyl-CoA carboxylase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenol-pyruvate carboxykinase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SMVT</td>
<td>Sodium-dependent multivitamin transporter</td>
</tr>
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</table>
Biotin is a water soluble vitamin that is synthesized by plants and several microorganisms. Mammals cannot synthesize biotin, therefore they rely on biotin intake through the diet and on microbial synthesis in the gastrointestinal tract to meet their biotin needs. When the needs of biotin are not met through the diet and microbial synthesis, characteristic symptoms of clinical biotin deficiency, such as dermatitis and alopecia, are manifested. Biotin deficiency can also affect foot health (mainly hoof integrity) in farm animals (38). While hoof integrity can be impaired when biotin deficiency is induced (38), hoof integrity can be improved with biotin supplementation (29,37,38,73,182).

Biotin requirements for lactating dairy cows have not been established (122). Because biotin is supplied in the feed and synthesized by microorganisms in the gastrointestinal tract (93,183), clinical biotin deficiency is unlikely to occur in ruminants. However, while evaluating the effect of biotin supplementation on foot health, field studies (16,98) observed that biotin supplementation also increased milk yield (Table 1.1). This response was later confirmed in controlled studies (90,92,198). The response of milk yield to biotin supplementation
suggests that biotin supply may not always be adequate for optimal biotin nutritional status and, therefore, optimal performance.

Even though biotin supplementation increased milk yield in several studies, this effect was not consistent. From nine comparisons in which the effect of biotin supplementation was tested against its control (≥20 vs. 0 mg/d), four did not observe a significant increase in milk yield (Table 1.1). Because the mechanism by which biotin supplementation increases milk yield is not known, it is still unclear why this effect varied among studies (90,92,145,198). Zimmerly and Weiss (198) reported a linear increase in milk yield when cows were supplemented with 0, 10 and 20 mg/d, suggesting that biotin supply from the feed and microbial synthesis can be limiting. Majee et al. (90) observed that an increase in dry matter (DM) intake accompanied the increase in milk yield in cows supplemented with biotin. This observation is unique among studies evaluating the effect of biotin supplementation (Table 1.1). Whether the increase in milk yield was related to DM intake rather than to biotin supplementation is unclear. The response in milk yield to biotin supplementation may also be dependent on production potential, as biotin did not increase milk yield when supplemented to low-producing (<20 kg/d) dairy cows (51). Our poor understanding on how biotin supplementation affects milk yield is substantiated by results from Rosendo et al. (145), who reported that biotin did not increase milk yield by high-producing (≥35 kg/d) cows. Even less clear is why biotin increased milk yield when supplemented (20 mg/d) alone, but not when supplemented (20 or 40 mg/d) accompanied with a B-vitamin blend (90).
An increase in milk yield may have great economic implications for the dairy industry. The increase in milk in response to biotin supplementation averaged $1.8 \pm 0.7$ kg/d (Table 1.1). Considering that the state of Ohio has approximately 260,000 dairy cows (1), total Ohioan farmer’s gross income$^1$ may increase by $43$ million per year. The cost of supplementing biotin (20 mg/d) in a 305-d lactation would be approximately $12$ per cow. Therefore, the net increase in total Ohioan farmer’s income would be approximately $40$ million per year. However, this estimate considers that all lactating cows would be responsive to biotin supplementation in every day of their lactation. Based on the variable response from reported studies, these assumptions are likely inaccurate. Therefore, further research should focus on understanding how and under which circumstances biotin supplementation increases milk production.

The overall objective of this research was to enhance our understanding of when and how biotin supplementation increases milk production by lactating dairy cows. The difficult quantification of biotin in feeds and biological fluids and the lack of a sensitive determinant of biotin status are two major reasons why our understanding is limited. Concentrations of avidin-binding substances (ABS) in blood and milk have been used as measures of biotin status in lactating dairy cows (145,198). In addition to biotin, ABS includes biotin analogues that might not have vitamin activity. The first objective was to evaluate different measures of biotin status in cows producing different quantities of milk. In addition to the typical measures (i.e., concentrations of ABS in blood and milk), the urinary

$^1$ Assuming a Class I milk price equal to 14 $/cwt and a 305-d lactation.
excretion of 3-hydroxyisovaleric acid (3HIA) was evaluated. Finally, biotin is a cofactor of two biotin-dependent enzymes, pyruvate carboxylase (PC) and propionyl-CoA carboxylase (PCC), which are involved in gluconeogenesis. The second objective of this research was to evaluate the effect of biotin supplementation on the activities of PC and PCC in the liver of lactating dairy cows.
<table>
<thead>
<tr>
<th>Study</th>
<th>Dose (mg/d)</th>
<th>Days after parturition</th>
<th>DM Intake (kg/d)</th>
<th>Milk Yield (kg/d)</th>
</tr>
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<tr>
<td>Midla et al. (98)</td>
<td>0</td>
<td>25 to 293</td>
<td>NR</td>
<td>38.7\textsuperscript{b}</td>
</tr>
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<td>✓</td>
<td>39.7\textsuperscript{a}</td>
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<td>NR</td>
<td>NR</td>
<td>18.5</td>
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<td>✓</td>
<td>17.3</td>
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<td>Zimmerly and Weiss (198)</td>
<td>0</td>
<td>1 to 98</td>
<td>19.4</td>
<td>36.9\textsuperscript{a}</td>
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<td></td>
<td>10</td>
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<td>19.8</td>
<td>37.8</td>
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<td>39.7</td>
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<td>1 to 120</td>
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<td>22</td>
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<td>NR</td>
<td>33.1\textsuperscript{b}</td>
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<td>✓</td>
<td>34.6\textsuperscript{a}</td>
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<td>46 to 158</td>
<td>25.0\textsuperscript{b}</td>
<td>37.2\textsuperscript{b}</td>
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<tr>
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<td>20</td>
<td>✓</td>
<td>25.7\textsuperscript{a}</td>
<td>38.9\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>20 + 1×BV\textsuperscript{3}</td>
<td>✓</td>
<td>25.0\textsuperscript{b}</td>
<td>38.3\textsuperscript{ab}</td>
</tr>
<tr>
<td></td>
<td>40 + 2×BV</td>
<td>✓</td>
<td>24.4\textsuperscript{b}</td>
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<td>84 to 168</td>
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<td></td>
<td>30</td>
<td>✓</td>
<td>23.6</td>
<td>34.8</td>
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\textsuperscript{1} NR = not reported.
\textsuperscript{2} Linear effect for milk yield.
\textsuperscript{3} BV = B-vitamin blend.
\textsuperscript{a,b,c} Rows within studies with different superscripts differ (P < 0.05).

**Table 1.1.** Effect of biotin supplementation in milk yield by lactating dairy cows.
CHAPTER 2

LITERATURE REVIEW

“Little is taught today as to how each of our particular areas of the biological sciences developed. For many students the 'important stuff' now goes into the past for only 7-8 years.” M. D. Lane (84).

Discovery of the Protective Factor against Egg White Injury

Biotin, also known as vitamin H or vitamin B₈, was discovered at the beginning of the 20th century when several studies were performed to understand why adverse effects were observed in animals fed diets containing raw egg white or "egg albumin" as the sole protein source. Bateman (12) observed that rats consuming diets containing raw egg white (as either fresh or dried form) had a greater incidence of diarrhea than rats consuming diets containing cooked egg white. Boas (21) observed that rats consuming diets containing dried egg white as the sole protein source showed weight loss and dermatitis. This dermatitis was referred as a “pellagra-like disorder” characterized mainly by roughness and thinness of the hair particularly around the mouth, nose and eyes, redness and swelling of the lips, paws and bared areas of the skin, dark pigmentation and
even skin hemorrhages in severe cases (21,130,131). Based on the observations from Boas, Parsons (130) evaluated the incorporation of liver powder (5 to 20% DM basis) into purified diets containing a high proportion of dried egg white (66% DM basis) as a protection against egg white injury in rats. The author not only observed a dramatic decrease in rat mortality (90 and 7% for control and liver-containing diets, respectively), but also a delay in the appearance and a decrease in the severity of dermal symptoms. These observations were the first indicators of the existence of a protective factor against egg white injury. In 1931 (as cited by György, 58), Paul György named this factor vitamin H (from haut, which means skin in German; 11).

To evaluate concentrations of vitamin H, György (58) determined the capabilities of different foodstuffs to cure egg white injury in rats and concluded that the main sources of vitamin H were liver, kidney, yeast, and cow’s milk. These observations did not simply give information about sources of vitamin H, but also were the beginning of a series of studies showing that vitamin H is a water-soluble compound (58) with acidic properties (19) that can be extracted and precipitated (58), and therefore obtained in concentrated forms.

Before the discovery of vitamin H, Kögl and Tommis (as cited by West and Wilson, 186) isolated a compound from egg yolk that was named biotin. Contemporary to the studies of György and coworkers, other studies evaluated the effect of certain factors on microbial growth. Allison et al. (4) reported a specific factor obtained from sugar cane that is essential for respiration by \textit{Rhizobium trifolii}, the \textit{N}_2-fixating bacteria present in red clover (\textit{Trifolium}
pratense). These authors named this factor coenzyme R (R referring to respiration). Based on the growth of Rhizobium trifolii, West and Wilson (186) concluded that coenzyme R is identical to biotin, the growth factor extracted from yeast and egg yolk. Snell et al. (163) compared the growth of Saccharomyces cerevisiae when cultured with a liver extract or with Kögl's biotin. Based on similar growth patterns of Saccharomyces cerevisiae and on the similar chemical and physical properties of biotin and the substance of interest, the authors concluded that the active principle in liver extract was biotin.

György et al. (61) realized that some of the chemical and physical properties of vitamin H were similar to those of coenzyme R and biotin and therefore hypothesized that vitamin H was the same compound as coenzyme R and biotin. To test this hypothesis, György et al. (61) first submitted vitamin H preparations to Dr. Allison's laboratory to determine coenzyme R activity and observed that vitamin H had substantial coenzyme R activity. They concluded that vitamin H and coenzyme R were identical or closely related compounds. Du Vigneaud et al. (43) observed that rats suffering egg white injury were cured after subcutaneous injection of Kögl’s biotin and concluded that vitamin H, the protective factor against egg white injury, and biotin were the same compounds.

The mechanism by which biotin served as a protective factor against egg white injury started to be elucidated in the early 1940’s, when Eakin et al. (47) observed that the concentration of biotin in tissues (e.g., liver and kidneys) was less for chicks consuming a diet containing dried egg white than for chicks consuming a diet containing casein as the protein source. Because it was
measured in both diets, the authors suggested that biotin from the diet was not available for the tissues in chicks consuming the diet containing dried egg white. In contrast to the theory of a direct toxic effect of egg white (130), Eakin et al. (47) hypothesized that egg white makes biotin unavailable and that this can be overcome by offering excessive amounts of biotin in the diet. In a later study, Eakin et al. (46) demonstrated that a constituent of raw egg white inactivates biotin making it unavailable for yeast growth. György et al. (62) evaluated whether avidalbumin, a constituent of egg white, is related to egg white injury. For this, rats were fed a diet containing cooked dried egg white with or without the addition of avidalbumin concentrates. Results showed not only that inclusion of avidalbumin results in the typical syndrome of egg white injury, but also that rats consuming diets without avidalbumin recovered from this syndrome. From these observations they concluded that avidalbumin is the toxic constituent of egg white, and that this toxic effect is attributed to the fixation or binding of biotin to avidalbumin that results in the unavailability of biotin for absorption. Avidalbumin (literally hungry albumin) was later named avidin (48).

**Biotin Structure**

The structure of biotin was elucidated in the early 1940’s by Vincent Du Vigneaud and co-workers at Cornell University (17). This accomplishment started after crystalline biotin was obtained by Du Vigneaud et al. (44). In addition to determining the composition of biotin (i.e., C_{10}H_{16}O_{3}N_{2}S, with a molecular weight
of 244.3), this event allowed Du Vigneaud et al. (44) to determine that biotin is a monocarboxylic acid.

Since the first studies of egg white injury, biotin was believed to be an \( \alpha \)-amino acid (19, 24, 163). This postulate was discarded after Brown and Du Vigneaud (24) observed that yeast growth activity was not affected after subjecting biotin to ninhydrin (a potent reactant with \( \alpha \)-amino acids). Hofmann et al. (66) subjected biotin to strong alkali and isolated a compound containing two amino groups and a melting point different from biotin. This compound was referred to as diaminocarboxylic acid (Figure 2.1). Because it was the starting compound for many other experiments, the discovery of diaminocarboxylic acid, in addition to the isolation of crystalline biotin, may be considered one of the most relevant events in the elucidation of the structure of biotin.

Diaminocarboxylic acid contained one carbon atom and one oxygen atom less and two hydrogen atoms more than biotin (i.e., \( \text{C}_9\text{H}_{18}\text{O}_2\text{N}_2\text{S} \)). Based on this composition, Hofmann et al. (66) suggested that the cleavage of a cyclic urea derivative would likely result in such a change in composition. Melville et al. (95) further hypothesized that, if Hoffmann’s postulate is true, diaminocarboxylic acid should be converted back to biotin through reactions utilized to synthesize urea derivatives (Figure 2.2). After subjecting diaminocarboxylic acid to carbonyl chloride (which converts amines to urea), Melville et al. (95) obtained a crystalline compound with a melting point similar to that of natural biotin. When tested by yeast growth, they also observed that the activity of the synthetic biotin
was similar to that of natural biotin. These observations validated the cyclic urea structure in the molecule of biotin (66).

Hofmann et al. (66) observed that the sulfur atom of biotin was stable and postulated a thioether structure within the biotin molecule. A previous study of Brown and Du Vigneaud (24) showed that the activity of biotin (determined by yeast growth) was completely destroyed when biotin was subjected to 5% hydrogen peroxide. Hofmann et al. (66) observed that two oxygen atoms were added to the molecule without loss of any carbon or hydrogen atoms (i.e., $\text{C}_{10}\text{H}_{16}\text{O}_5\text{N}_2\text{S}$) after subjecting biotin to 5% hydrogen peroxide. Hofmann et al. (66) observed that this product had a greater melting point than biotin and a behavior typical of sulfones (Figure 2.3). After discarding the possibility for the existence of double bonds (i.e., potential site for oxygen incorporation), Hofmann et al. (66) postulated that hydrogen peroxide results in the oxidation of a thioether to a sulfone (Figure 2.3).

Based on the functional groups, the absence of double bonds and on the hydrogen to carbon ratio, Hofmann et al. (67) suggested that the molecule of biotin should contain a two-ring structure. Kögl and co-workers (as cited by 67) also suggested that sulfur is part of one of these rings. Partial evidence to this two-ring structure was given by Hofmann et al. (68), who demonstrated that biotin contained a 5-membered urea ring structure (Figure 2.4).

While attempting to elucidate the structure of the carbon skeleton, Hofmann et al. (67) observed that adipic acid is obtained from the oxidation of diaminocarboxylic acid (Figure 2.5). Du Vigneaud et al. (42) and Hofmann et al.
demonstrated that one of the carboxyl groups of adipic acid is the original carboxyl group of biotin and suggested that biotin may contain either an n-valeryl or an n-butyryl side chain attached to one of the rings. After demonstrating that dethiobiotin contains only one methyl group (42) and that thiophenevaleric acid can be formed from biotin (97), Du Vigneaud and co-workers defined the structure of biotin as 2’-keto-3,4-imidazolido-2-tetrahydrothiophene-valeric acid (Figure 2.6).

In addition to revealing the structure of biotin, some of the events in the elucidation of the structure of biotin have considerable relevance in the understanding of some aspects of biotin quantification and biotin catabolism. For example, the urea ring structure is the portion of biotin that interacts with avidin. The presence of this or a similar structure explains the cross-reactivity of biotin analogues in avidin-binding assays for the quantification of biotin. The oxidation of the sulfur atom when exposed to hydrogen peroxide indicates that biotin is susceptible to oxidizing conditions. Oxidizing conditions may accelerate the catabolism of biotin possibly resulting in biotin deficiency (160).

**Biotin Quantification**

Several procedures exist for the quantification of biotin. Despite the existence of these methods, the quantification of biotin in tissues, feeds and biological fluids is difficult. Lack of specificity (i.e., biotin vs. biotin analogues) and low sensitivity are two major reasons for the difficulty in the quantification of biotin in feeds and biological fluids.
Biological Assays

Biological assays are based on the capabilities of different feeds (47,58,130) or fluids (93,163) to cure egg white injury. For this assay, the survival index or growth of animals consuming a diet containing an unknown concentration of biotin is compared to the survival index or growing performance of animals consuming a diet containing a known concentration of biotin. The basal diet contains raw egg white as the main protein source to induce biotin deficiency. Rats (58,130) and chicks (47) are typically used for this type of assay.

The greatest advantage of this assay is that it reflects the bioavailability and activity of biotin for the animal disregarding the presence of biotin analogues, such as bisnorbiotin or biocytin. One major disadvantage of this assay is that the concentration of biotin in the feedstuff of interest may be overestimated due to the synthesis of biotin by microorganisms from the gastrointestinal tract. This assay is also expensive and labor intensive, and requires a long period of time to process a sample (58). A unit was considered as the daily dose of a foodstuff that in four weeks completely cures egg white injury (58). Animal welfare issues may also discourage the use of biological assays for the quantification of biotin.

Microbiological Assays

Microbiological assays have been used for decades for the quantitative determination of biotin (2,10,163). These assays compare the growth rate of microorganisms cultured in a medium enriched with a source of unknown biotin concentration with the growth rate of microorganisms cultured in media with a known biotin concentration. Production of specific metabolites by certain
microorganisms (e.g., lactate production by *Lactobacillus* sp.) can also be used as an indirect measure of microbial growth (189). Different bacteria (189), yeast (124,163) and fungi (178) have been used for this type of assay.

Some advantages of microbiological assays, when compared to biological assays, are the increased sensitivity and the reduced period of time required to process a sample (62,163). One disadvantage of these assays is the possible lack of specificity for biotin. Nielsen et al. (124) observed that the growth of *Saccharomyces cerevisiae* was reduced, but not inhibited (i.e., 80% of control), when peroxide-treated biotin (presumably biotin sulfoxide) was added to the media. Dittmer et al. (41) reported similar growths of *Saccharomyces cerevisiae* when cultured with biotin or dethiobiotin. Contrary to the observations for *Saccharomyces cerevisiae*, Nielsen et al. (124) and Dittmer et al. (41) showed that the growth of *Lactobacillus casei* was totally inhibited when cultured with peroxide-treated biotin and dethiobiotin, respectively. Microbiological assays can also be considered labor intensive, making these unattractive assays when large number of samples needs to be processed.

*Chromatography Methods*

The concentration of biotin in certain samples can be directly determined by chromatography methods (33,70). Janecke and Voege (70) developed a gas liquid chromatography (GLC) method for the detection and quantification of derivatized biotin, biotin sulfoxide and biotin sulfones. Chastain et al. (33) reported a high-performance liquid chromatography (HPLC) method for the
quantification of biotin and biotin analogues (e.g., biocytin, dethiobiotin, bisnorbiotin, tetranorbiotin, biotin sulfoxide and biotin sulfone).

The greatest advantage and disadvantage of these methods are their great specificity and their poor sensitivity, respectively. Chastain et al. (33) reported a lower limit of detection of 40 nmol/mL (UV detector). Concentrations of biotin in blood (110,198), urine (109,132) or milk (108,198) are typically less than 10, 400 and 300 pmol/mL, respectively. Due to their poor sensitivity, the use of these methods is limited for samples with pharmacological concentrations of biotin.

**Avidin-binding Assays**

Avidin-binding assays are indirect methods commonly used for the quantification of biotin (101,155,198). The principle of these assays lies in the great affinity of avidin for biotin (48,57). The multiple sites of avidin for binding biotin (14) are also an important characteristic for this type of assay. Various avidin-binding assay protocols exist, and most of them are based on the quantification of enzymes (e.g., horseradish peroxidase) linked to biotin (155) or avidin (101).

The greatest disadvantage of avidin-binding assays is their poor specificity. The imidazolidone ring is the structure recognized by the binding-sites of avidin (57). Avidin has considerable affinity for many substances containing a structure identical to the imidazolidone ring of biotin (40,190,192). Dittmer and Du Vigneaud (40) observed that the growth of *Saccharomyces cerevisiae* was not dramatically inhibited when biotin was added to a culture medium previously
incubated with avidin and imidazolidone aliphatic acids. This observation suggested that the binding of avidin to imidazolidone aliphatic acids reduced or inhibited the binding of avidin to biotin. Using similar protocols, Wright et al. (190,192) demonstrated the binding of avidin to biocytin and 2,3-ureylene-cyclohexyl-valeric acid. Avidin also has affinity for substances containing a structure similar, but not identical, to the imidazolidone ring of biotin (25). Brown and Du Vigneaud (25) reported that the biotin activity of a thiourea analogue of dethiobiotin for the growth of *Saccharomyces cerevisiae* was totally inhibited when avidin was added to a medium. The non-specific binding of avidin to biotin can result in an overestimation of the concentration of biotin when using avidin-binding assays.

To avoid overestimations in biotin quantification, Mock and co-workers (100-102) proposed a two-step procedure that allows the specific quantification of biotin and biotin analogues. This procedure consists of the quantification of avidin-binding activity in the chromatographic fractions after the separation of biotin and biotin analogues by HPLC (33,102). By using the two-step procedure, Mock et al. (109) observed that biotin accounted for only 43, 56 and approximately 70% of the total avidin-binding activity (molar basis) in urine, serum and milk from humans, respectively, whereas most of the remaining avidin-binding activity was attributed to bisnorbiotin and biotin sulfoxide (*Figure 2.7*). One disadvantage is that procedure may be considered labor intensive and time consuming (132).
A Potential GLC Protocol for the Quantification of Biotin

Chromatography methods measure directly and specifically biotin and biotin analogues (33,70). These are attractive methods for measuring biotin concentration in biological fluids except for their poor sensitivity. Janecke and Voege (70) demonstrated that biotin and biotin analogues can be derivatized for their separation and detection using GLC. Because of this, in addition to the simplicity of the equipment and to the low maintenance costs, a GLC procedure would be an attractive procedure for measuring biotin in biological fluids.

The sensitivity of a GLC procedure may be improved by modifying the sample of interest, by changing the detector or by a combination of these two. Concentrating samples may increase the concentration of the analyte to values above the detection limit of the detector. Based on the reported detection limits for biotin (33), samples would need to be concentrated at least 10-fold. Changing the detector may also improve the detection limit for certain analytes. For instance, for halogenated compounds the detection limit can be lower when using an electron-capture detector than when using a flame-ionization detector (91). Aprea et al. (5) used a GLC procedure with an electron-capture detector for measuring the concentration of derivatized pyrethroid metabolites in urine and reported a detection limit as low as 3 pmol/mL. Biotin and biotin analogues are not halogenated compounds. However, if biotin and biotin analogues could be derivatized with a highly-halogenated compound such as pentafluorobenzyl bromide (5,91), the detectability of these compounds by an electron-capture detector might be increased dramatically. If this was possible, a GLC procedure
using electron-capture detector may result in a method for the direct and specific quantification of biotin and biotin analogues.

**Free and Protein-bound Biotin**

Early studies found that a fraction of the total biotin in different extracts was bound to proteins (58,60,163,174). Protein-bound biotin in animal and plant tissues is typically covalently linked to the ε-amino group of a lysine residue of the tetrapeptide ALA-MET-LYS-MET of biotin-dependent carboxylases (26,82). This biotinyl-lysine conjugate is commonly known as biocytin. Biotin is also bound to non-carboxylase proteins in seeds (45). In seed proteins, biotin is linked to the ε-amino group of a lysine residue of a peptide sequence different than that of biotin-dependent carboxylases (71).

The proportion of protein-bound biotin is variable among samples. Protein-bound biotin accounted for 85, 13 and less than 5% of the total biotin in liver (174), plasma (103) and milk (108), respectively. In plants, the proportion of the total biotin that is protein-bound varies across tissues and also within tissues depending on maturity stage. Baldet et al. (10) reported that approximately 20% of the total biotin in green pea leaves is bound to proteins. A negligible proportion of protein-bound biotin was observed in immature pea seeds, but protein-bound biotin accounted for 83% of the total biotin in mature pea seeds (45). Bryden (28) reported that protein-bound biotin accounted for 60% of the total biotin in cecal digesta from birds.

Biotin bound to proteins can be released by chemical or enzymatic hydrolysis before biotin quantification. For chemical methods, samples are
subjected to acid hydrolysis (1 to 3 M H$_2$SO$_4$) to release protein-bound biotin (10,27,108,174). Mock et al. (108) observed that incubations in H$_2$SO$_4$ substantially increase the degradation of biotin (12 to 37%), likely due to its oxidation to biotin sulfoxide. To evaluate the liberation of protein-bound biotin, Mock et al. (108) incubated bovine serum albumin (BSA) with biotin to form biotinyl-BSA, and observed that incubating milk samples (i.e., protein precipitates) with 2 M HCl releases more than 95% of the protein-bound biotin with less than 10% biotin degradation. This procedure was later used for other foods (166). Biocytin is also hydrolyzed by the enzyme biotinidase (77,174). Details of the action of biotinidase are discussed later (see Biotin Absorption section).

The approach in the determination of total biotin may affect interpretations dramatically. Recently, the Canadian group headed by Dr. Girard (155) proposed a procedure to determine total biotin, in which samples are incubated with an extract from pancreas before biotin quantification by an avidin-binding assay. The rationale for this procedure relies on the liberation of biotin from biocytin by the pancreatic enzyme biotinidase. Several concerns arise from their procedure and their interpretations. The hydrolysis of biocytin would not be absolutely necessary if the affinity of avidin to biocytin is similar to that of biotin. Their claim that biotinidase is the only enzyme known to hydrolyze biocytin (155) is unclear, but should not be interpreted as biocytin cannot bind avidin. The company R-Biopharm (Germany) commercializes a kit for the quantification of biotin by a competitive enzyme binding assay (i.e., avidin-binding assay). Based on their
specifications, biocytin has 83% cross-reactivity with avidin. In contrast, Santschi et al. (155) claimed (from unpublished data) that “without treatment with pancreas extract, biocytin was not detected by the assay.” The reasons for this lack of binding activity are not clear. Whether other proteases from pancreatic extracts, and not biotinidase, are required for an appropriate quantification of total biotin is uncertain. However, the high total biotin concentration reported by Santschi et al. (155) raises some concerns for this procedure. Biotin concentrations (DM basis) in foods, plant tissues and swine and ruminant diets were summarized (Table 2.1). Biotin concentration of whole egg, a biotin-rich food, is 1.5 mg/kg. When excluding data from Santschi et al. (155), biotin concentration in milk, in plant tissues and in swine and ruminant diets averaged 0.24 mg/kg and ranged from 0.1 to 0.4 mg/kg. The biotin concentration for a ruminant diet reported by Santschi et al. (155) is not only greater than these typical values, but also greater than that of whole eggs, one of the richest sources of biotin (166). Great proportions of protein-bound biotin (i.e., biocytin) might explain this high value. However, assuming a concentration of free biotin equal to 0.5 mg/kg (value equal to the average plus two SD for samples other than whole eggs; Table 2.1), the concentration of protein-bound biotin would need to be approximately 6.5 mg/kg to obtain a concentration of total biotin equal to 7.0 mg/kg. In other words, protein-bound biotin would account for more than 90% of the total biotin. Whether or not this is true in ruminant diets is uncertain based on the limited data. However, based on data for total biotin (10,27,98) summarized in Table 2.1, the concentration of total biotin reported by Santschi et
al. (155) is likely overestimated. Santschi et al. (155) attributed these greater values to the greater specificity of their procedure. More specifically, they claimed that “microbiological assays are less specific than the chemical methods used in the present experiment.” Several concerns arise from this claim. First, microbiological assays may be non-specific depending on the organism utilized. As discussed above, *Lactobacillus casei* grows in biotin-containing media, but not in dethiobiotin-containing media (41), suggesting that microbiological assays can be specific for biotin. Second, total biotin can be determined by microbiological assays as long as samples are previously subjected to chemical hydrolysis (10,27,108,166). Finally, avidin has affinity for several biotin analogues other than biocytin (e.g., dethiobiotin, bisnorbiotin and biotin sulfoxide). The method of Santschi et al. (155) does not separate biotin from biotin analogues by chromatography methods (158). Therefore, the avidin-binding assay utilized by Santschi et al. (155) would not be specific for biotin as they implied. Avidin can bind several substances as discussed previously. The overestimation of total biotin concentration by Santschi et al. (155) suggest that interfering substances may have been created, perhaps, as a result of the incubation with an extract from the pancreas. It is worth mentioning, however, that biotin degradation occurs during acid hydrolysis (108), and therefore total biotin may have been underestimated in previous methods (10,27,108,166).

**Conclusion**

In conclusion, a technique for an accurate quantification of biotin is still needed. Studies comparing microbiological and avidin-binding assays are also
needed. The procedure used for the release of protein-bound biotin can dramatically affect biotin quantification. Whether hydrolysis of protein-bound biotin should be performed with HCl (108) or with pancreatic extracts (155) deserves further evaluation. Currently, avidin-binding assays after separation of biotin analogues from biotin by chromatography methods seem to be the most appropriate method for quantification of biotin in feeds and biological fluids (101,166).

**Biotin Synthesis**

The pathway of biotin synthesis has been studied in plants, bacteria and yeast. Biotin is synthesized from pimelic acid and alanine through four enzymatic steps (Figure 2.8). Even though the exact precursors for the synthesis of pimelic acid are still not known, evidence exists that pimelic acid can be synthesized from long-chain fatty acids (125) and from acetate (157). The first step of this pathway involves the decarboxylative condensation of alanine with pimeloyl-CoA to produce 7-keto-8-amino pelargonic acid (KAPA). This reaction is catalyzed by the enzyme KAPA synthase, which requires pyridoxal-5-phosphate as a cofactor (133). The second step involves the amination of KAPA to produce 7,8-diamino pelargonic acid (DAPA). This amino transfer is catalyzed by the enzyme DAPA synthase, which also requires pyridoxal-5-phosphate as a cofactor and S-adenosyl-methionine as an amino donor (167). The third step involves the carbonylation of DAPA to produce dethiobiotin. This reaction requires ATP and is catalyzed by the enzyme dethiobiotin synthase (167). The last step involves the
addition of a sulfur atom into dethiobiotin to produce biotin. This reaction is catalyzed by the enzyme biotin synthase (18,49,127), which requires pyridoxal-5-phosphate as a cofactor (127). A study using $^{35}$S suggests that cysteine is likely the S donor in this reaction (18). Biotin synthase also contains a cluster containing Fe and S that mediates the reductive cleavage of S-adenosyl-methionine, releasing deoxyadenosyl radical, which activates and breaks the C–H bonds at the 6 and 9 position of dethiobiotin (18,49). In addition, several cofactors, such as flavodoxin, flavodoxin reductase and NADPH, appear to be involved in this reaction (18,49).

It is widely accepted that biotin is synthesized by the microbes of the gastrointestinal tract (78,93,124,132,183). McElroy and Jukes (93) evaluated the inclusion of dried rumen contents on the development of egg white injury in chicks. Chicks were fed a basal diet deficient in B-vitamins (negative control), the basal diet supplemented with a ruminant diet deficient in B-vitamins or the basal diet supplemented with rumen contents from a cow consuming the ruminant diet deficient in B-vitamins. None of the chicks fed the basal diet supplemented with rumen contents manifested symptoms of egg white injury, while 70% of the chicks consuming either the basal diet alone or the basal diet supplemented with the ruminant diet manifested symptoms of egg white injury. Wegner et al. (183) observed that the concentration of biotin in rumen contents was greater than that of the basal diet and attributed this observation to the synthesis of biotin in the rumen. Nielsen et al. (124), Kopinski et al. (78) and Peterson et al. (132) performed metabolic studies in rats, pigs and ewes, respectively, and observed
that the excretion of biotin in urine and feces was greater than biotin intake. All these observations supports that biotin is synthesized in the gastrointestinal tract.

Many aspects of biotin synthesis by the gastrointestinal microbes are not clearly understood. For instance, it is widely accepted that microbes from the rumen synthesize biotin (2,93), although information on the specific microorganisms that synthesize biotin is limiting. In a review, Wolin and Miller (188) summarized that two out of nine rumen bacteria do not require biotin for their growth. Although the rumen bacterial population is obviously not limited to nine species, it is surprising that only a few of these rumen bacteria are biotin autotrophs. Burkholder and McVeigh (30) also reported that five out of six intestinal bacteria synthesize biotin. With a different approach, Landy et al. (83) observed that six out of thirteen bacteria found in the gastrointestinal tract could grow in an avidin-containing medium (Table 2.2) and attributed this to their capability to synthesize biotin. The latter studies (30,83) show that a more diverse population of gastrointestinal bacteria is capable of synthesizing biotin.

The information available for biotin synthesis by ruminal and intestinal bacteria might be misinterpreted based on the methodologies used. Some of the information of biotin requirements for rumen bacteria was obtained from in vitro studies in which bacterial strains were cultured using biotin-free media (159). However, because an organism cannot grow in biotin-free media should not be conclusive evidence that the organism cannot synthesize biotin. In the early days of the discovery of biotin, the growth of *Saccharomyces cerevisiae* was used as a determinant of biotin content in different samples (163). This procedure was
based on the requirement of *Saccharomyces cerevisiae* for biotin. However, Dittmer et al. (41) reported that dethiobiotin was as effective as biotin in stimulating the growth of *Saccharomyces cerevisiae*. This information suggests the either dethiobiotin has biotin-like activity or that dethiobiotin was converted to biotin. The later possibility suggests that *Saccharomyces cerevisiae* has the capability of synthesizing biotin (i.e., expresses the gene for biotin synthase) when the substrate (i.e., dethiobiotin) is present. Dittmer et al. (41) observed that dethiobiotin lacks biotin-like activity for *Lactobacillus casei*. However, cell extracts from *Saccharomyces cerevisiae* previously cultured with dethiobiotin had growth-promoting activity for *Lactobacillus casei*. Dittmer et al. (41) concluded that dethiobiotin was likely converted to biotin by *Saccharomyces cerevisiae*. This information suggests that *Saccharomyces cerevisiae*, an organism that requires biotin for its growth (163), has the appropriate enzyme to convert dethiobiotin into biotin. In future studies, the use of molecular techniques to determine the expression of genes and the synthesis of enzymes involved in biotin synthesis accompanied by pure and mixed cultures in media containing the precursors for biotin synthesis would substantiate the information on the capability of specific rumen microbes to synthesize biotin.

Conflicting interpretations exist among studies about the quantity of biotin synthesis in the gastrointestinal tract. Net biotin synthesis in the rumen is typically determined as the difference between biotin flow to the duodenum and biotin intake, whereas net synthesis in the small intestine is typically determined as the difference between biotin flow out of the ileum and biotin flow to the
duodenum (99,155,199). Net biotin synthesis in the rumen (ranging from 0.1 to 2.4 mg/d) was reported for beef steers consuming 5 to 7.5 kg DM (99,199), whereas no net biotin synthesis or net biotin utilization in the rumen (ranging from 0 to 15 mg/d) was reported for lactating dairy cows consuming 18 to 22 kg DM (155,158). In addition to the different type of animal, these two groups of studies used different methods for the quantification of biotin. While Miller et al. (99) and Zinn et al. (199) used microbiological assays, Santschi et al. (155) and Schwab et al. (158) used an avidin-binding assay after hydrolysis with pancreatic enzyme. Whether the different results are related to animal type or to analytical methodologies remains unclear. By kinetic analysis of biotin in blood, Frigg et al. (54) suggested that ruminal synthesis did not supply absorbed biotin. This conclusion was based on assumed (but not measured) biotin concentrations in feeds and biotin availabilities from feeds. Biotin concentration of feeds is critical to estimate biotin intake and, therefore, to estimate net biotin synthesis. Because of the indirect methodology and the cited assumptions, their conclusion (54) regarding net biotin synthesis in the rumen might not be reliable. Based on the limited data and the mixed results, I can only conclude that the quantity of biotin synthesized in the rumen it is still not known.

The intestine is a major site of microbial biotin synthesis. Biotin flows out of the ileum greater than biotin flows to the duodenum were observed in steers (99) and pigs (79), suggesting that net biotin synthesis occurs in the small intestine. Kopinski et al. (79) also observed that the flow of biotin decreases in the first portion of the small intestine, but increases in the second portion of the
small intestine, suggesting that biotin absorption occurs in the first part of the intestine, but also that net biotin synthesis occurs in the whole small intestine. Contrary to the observations of Miller et al. (99) and Kopinski et al. (79), Santschi et al. (155) observed in dairy cows that the flow of biotin out of the ileum was less than the flow of biotin into the duodenum, suggesting that absorption was greater than biotin synthesis in the small intestine. Finally, high concentrations of biotin were observed in feces of rats (124) and ewes (132), suggesting that large amounts of biotin are synthesized in the large intestine.

Information regarding the factors that affect biotin synthesis in the gastrointestinal tract is limiting (2,132,158). Using continuous cultures and a microbiological assay with acid hydrolysis, Abel et al. (2) estimated biotin microbial synthesis when the substrate contained different hay to barley grain ratios (from 83:17 to 17:83). Biotin balance (i.e., an index of net biotin synthesis) was estimated as the difference between the amount of biotin in the solid and liquid phases and the amount of biotin in the substrate. Biotin balance was similar when the substrate contained 17 to 67% barley, but decreased when the substrate contained 83% of barley. This observation suggests that at very high concentrations of grain, biotin synthesis by rumen microbes is decreased or that biotin degradation by rumen microbes is increased. Using a single-step avidin-binding assay, Peterson et al. (132) measured the output of biotin in feces and urine of ewes consuming diets containing different forage to concentrate ratios. The difference between biotin output and biotin intake in this study can be considered as an indirect index of biotin synthesis in the gastrointestinal tract.
The biotin balance was quadratically affected by the forage to concentrate ratio of the diet (Figure 2.9). This observation suggests that biotin synthesis increases when the proportion of concentrate in the diet increases from 5 to 77%. However, a greater increase in the proportion of concentrate in the diet (≥90% concentrate) seemed to have a negative effect on biotin synthesis, although this decrease may also be related to a lower DM intake. Schwab et al. (158) measured the flow of biotin to the duodenum of lactating dairy cows fed diets containing two different forage to concentrate ratios (35 vs. 60%) and two different concentrations of non-fiber carbohydrates (30 vs. 40%) and observed for all diets that biotin flow to the duodenum was less than biotin intake, suggesting no net biotin synthesis in the rumen. Using an avidin-binding assay without incubation with pancreatic enzyme, Santschi et al. (156) observed that the concentration of biotin in particle-free rumen fluid was greater (24.3 vs. 18.5 ng/mL) for cows consuming a high-forage diet than for cows consuming a low-forage diet (40:60 and 60:40 forage to concentrate ratio, respectively). The concentration of biotin in particle-associated and liquid-associated bacteria was similar among groups (156). Whether this information is of value is not clear given that they did not estimate microbial mass.

Little can be concluded from these conflicting and limited data (2,132,158). The decreased net biotin synthesis when the feed or substrate has very high proportions of concentrate (2,132) may have little significance given that these do not reflect typical diets for lactating dairy cows. Feeding diets with high concentrations of non-fiber carbohydrates did not affect ruminal biotin synthesis.
in lactating dairy cows (158). Based on sheep data (132), net biotin synthesis in the whole gastrointestinal tract may increase when the proportion of concentrate in the diets is increased to values similar to those of typical of diets for lactating dairy cows. Even though limited data suggest that diet does not affect biotin synthesis in the rumen (158), how net biotin synthesis is affected in the small and large intestines has not been evaluated in dairy cows.

**Biotin Absorption**

Biotin is absorbed in its free form; therefore, protein-bound biotin must be hydrolyzed for its absorption. The hydrolysis of biocytin is catalyzed by biotinidase, an enzyme found in pancreas, liver, kidney (174), blood (191), milk (126) and microorganisms (77). The activity of biotinidase is greatest at neutral or slightly acidic pH (77,174). Thoma and Peterson (174) observed that biocytin is not hydrolyzed by other gastric and intestinal enzymes, such as pepsin, trypsin, pancreatin, papain, rennin and amylase. Koivusalo et al. (77) observed that incubations of propionyl-CoA carboxylase (i.e., biotin-containing protein) with biotinidase alone did not release substantial amounts of biotin. Thoma and Peterson (174) also observed that biotinidase required the presence of other proteolytic enzymes (e.g., trypsin) to release protein-bound biotin from whole liver (174). These observations suggest that biotinidase alone cannot release biotin from biotin-containing enzymes.

Biotin has acidic properties and a pKa of 4.65 (154); therefore, biotin is typically present in its ionized form in the rumen and intestines (Table 2.3).
Consequently, biotin cannot be transported by passive diffusion into the cells of the gastrointestinal tract. In the stomach (pH < pKa) biotin is mainly present in its protonated form (Table 2.3). Whether biotin is absorbed by passive diffusion in the stomach is unknown. However, due to the fast digesta transit and to the reduced absorptive surface, biotin absorption in the stomach is probably minimal.

A facilitated mechanism in the brush-border membrane of the enterocytes allows the absorption of biotin against a concentration gradient in the intestine (149-152). This transport is sodium-dependent (149-152) and greatest under acidic conditions (152). Biotin absorption through this transporter can be decreased by biotin analogues (149-151) and pantothenic acid (148), suggesting that this transporter is not specific for biotin. The activity of the biotin transporter varies depending on the site within the intestine (152) and on biotin status (153). Using brush-border membrane vesicles, Said et al. (153) observed that biotin transport ($V_{max}$) increased in biotin-deficient rats and decreased in biotin-supplemented rats. To my knowledge, the presence of the biotin transporter in the rumen has not been reported and, therefore, needs to be determined.

Prasad et al. (135) transfected placental cDNA that encodes this sodium-dependent transporter into uterine cells and observed that the transport of biotin into these cells was substantially increased. This increase was inhibited by pantothenic acid and lipoic acid, but not by myoinositol, suggesting that the transporter is specific for biotin, pantothenic acid and lipoic acid. The increased transport of biotin and pantothenic acid was not observed in the absence of sodium, suggesting sodium-dependence. Prasad et al. (135) named this
transporter as sodium-dependent multivitamin transporter (SMVT). With a similar approach, Chatterjee et al. (34) found in rat intestine a variant of SMVT with the same functional characteristics. The gene for this SMVT was expressed in the duodenum, jejunum, ileum and colon (34).

The $K_m$ of SMVT in the intestine is in the micromolar range and varies depending on developmental stage (121). In recent years, another transporter for biotin was found in human peripheral blood mononuclear cells (PBMC; 195,196) and keratinocytes (56). This transporter is also sodium-dependent (195), but has a $K_m$ in the nanomolar range (195). In PBMC the transport of biotin by this transporter was not affected by pantothenic acid and lipoic acid (56,196), but was inhibited by biocytin (196). Balamurugan et al. (8) concluded that this high-affinity and biotin-specific transporter is not functional in intestine and liver cells.

In vitro studies show that the proximal small intestine is the main site of biotin absorptive activity (22,23,165). Using everted intestinal sacs from rodents, Spencer and Brody (165) observed that biotin is transported against a gradient in the proximal portion of the small intestine, while this transport decreased in the distal portion of the small intestine. Using intestinal loop in vivo techniques, Bowman et al. (23) and Bowman and Rosenberg (22) observed that biotin transport was greater in the jejunum than in the ileum of rats. Bowman and Rosenberg (22) also reported that the absorption of biotin in the proximal colon and cecum was considerably lower than in distal ileum and proximal jejunum. Said and Redha (149) observed that the transport of biotin in everted colonic sacs from rats is minimal, but substantial in jejunal and ileal sacs. Said et al.
observed that biotin transport in humans was greater in the duodenum than in the jejunum, and greater in the jejunum than in the ileum. Said et al. (153) observed that biotin transport in the jejunum of biotin-deficient rats was greater than that of control rats, which was greater than that of biotin supplemented rats. Various studies measured biotin absorption in vivo (28,79,80). Bryden (28) measured biotin flows in the intestines of birds and observed that 27% of the biotin intake was absorbed in the duodenum. Total biotin absorption at the distal ileum accounted for 60% of dietary biotin. Kopinski et al. (79) observed that 90% of the dietary biotin was absorbed in the proximal half of the duodenum of pigs. Kopinsky et al. (80) observed that the concentration of biotin in liver, kidney and heart tissues was increased after infusing biotin into the cecum of pigs, although they observed that this post-ileal absorption was less efficient than the absorption in the total gastrointestinal tract. Frigg et al. (53) observed that plasma concentration of biotin increased after an oral dose of 40 mg of biotin was given to dairy heifers, and that estimated bioavailability of synthetic biotin tended to be greater (41 vs. 55%, respectively) for control (i.e. non-supplemented) than for biotin-supplemented heifers. Although they made no distinction about the site of absorption, the estimated bioavailability of this dose averaged 48% (53). In lactating dairy cows, 25 to 46% of the biotin reaching the duodenum was absorbed in the small intestine (155).

The presence of a biotin transporter similar to SMVT in the gastrointestinal tract of dairy cows has not been determined. However, data from Majee et al. (90) might suggest the presence of an absorption system similar to the SMVT
previously described. Majee et al. (90) observed that biotin supplementation increased milk yield when biotin was supplemented alone, but not when biotin was supplemented accompanied by a B-vitamin blend. The authors suggested that negative interactions between biotin and other B-vitamins might have occurred (90). Chatterjee et al. (34) reported data suggesting that the uptake of biotin was 65% lower when cells were incubated with pantothenic acid (pantothenic acid to biotin molar ratio equal to 20) than when incubated without pantothenic acid. Interestingly, the B-vitamin blend used by Majee et al. (90) contained 26 moles of pantothenic acid per mol of biotin. Although totally speculative, if biotin uptake in dairy cows occurs by a SMVT transporter system similar to that previously described, a decrease in biotin uptake due to the presence of pantothenic acid in the B-vitamin blend might explain the results observed by Majee et al. (90).

In conclusion, a transport system is required for absorption of biotin. Biotin status can affect the activity of the transporter (153) and biotin bioavailability (53). Therefore this transporter may play an important role in maintaining biotin body homeostasis. Although present in non-ruminants, the presence of a transport system for biotin has not been determined in ruminants. Santschi et al. (155) and Schwab et al. (158) suggested that the decreased duodenal flow of biotin (relative to biotin intake) may be attributed to ruminal absorption of biotin, although no evidence exists to confirm this postulate. Determination of the presence of biotin transporters in the rumen epithelium would support that hypothesis.
Biotin Excretion

Three major routes exist for the elimination of biotin and biotin metabolites from the organism: urinary and biliary excretions and secretion into milk. In non-lactating animals, urinary excretion is the most important route of excretion (85,179). Lee et al. (85) injected intraperitoneally $[^{14}\text{C}]-\text{biotin}$ into rats fed a biotin-sufficient diet, and measured the radioactivity in urine, feces and expired air. Whereas negligible in feces and expired air, they found substantial amounts of radioactivity in urine. Wang et al. (179) observed that 98% of the total radioactivity injected intraperitoneally into rats as $[^{14}\text{C}]-\text{biotin}$ appeared in the urine. Biotin, bisnorbiotin and biotin sulfoxide, respectively, accounted for 93, 6 and 1% of the injected radioactivity (179), suggesting that biotin is not conjugated for its excretion.

The excretion of biotin in urine is regulated by a reabsorption mechanism (13,134). Podevin and Barbarat (134) and Baur et al. (13) observed in rabbits and rats, respectively, that the transport of biotin by brush-border vesicles is sodium-dependent and saturable with a $K_m$ in the micromolar range. Biotin reabsorption in the kidneys is not specific for biotin, but selective to major biotin metabolites (13,134). Baur et al. (13) reported that the transport of biotin into brush-border membrane vesicles from rat kidneys was substantially inhibited by dethiobiotin, and partially inhibited by bisnorbiotin (42 and 90% of control, respectively). The transport of biotin was not inhibited by biocytin (13,134), biotin sulfoxide and biotin sulfones (13). Recently, Balamurugan et al. (9) reported the presence of a biotin transporter in kidney cells that was sodium-dependent, had a
$K_m$ in the micromolar range, and was inhibited by dethiobiotin, pantothenic acid and lipoic acid. They concluded that reabsorption of biotin in kidney cells is regulated by a SMVT system, similar to that of intestinal cells.

Biotin excretion in urine can be affected by biotin status (9,85). Lee et al. (85) reported that biotin-deficient rats excreted less of the total $[^{14}\text{C}]$-biotin injected intraperitoneally than biotin-sufficient rats, suggesting that greater retention of biotin occurs in biotin-deficient rats. Mock et al. (107) observed that the daily urinary excretion of biotin decreased substantially when biotin-deficiency was induced to rats, whereas it increased in biotin-supplemented rats. The urinary excretion of biotin and biotin metabolites also decreased when marginal biotin deficiency was induced in humans (117). The reduction of urinary biotin excretion observed during biotin deficiency may be related to an increase in biotin reabsorption at the kidney level (9). Balamurugan et al. (9) reported that biotin uptake by kidney cells was greater when cultured in biotin-deficient media than when cultured in biotin-sufficient media. The increased uptake was related to an increased transcription of the gene for SMVT (9).

**Biotin Degradation**

While oxidation of the thioether converts biotin to biotin sulfoxide (94) and biotin sulfone (66), the loss of acetate units from the valeric acid side chain through a $\beta$-oxidation pathway converts biotin into bisnorbiotin and tetranorbiotin (69,146). Depending on the concentration of peroxide, biotin is converted into biotin sulfoxide (94) or biotin sulfone (66) when subjected to hydrogen peroxide.
In bacterial cell extracts, $[^{14}C]$-homobiotin was converted into $[^{14}C]$-norbiotin and $[^{14}C]$-trisnorbiotin (146) and $[^{14}C]$-biotin was converted into $[^{14}C]$-bisnorbiotin and $[^{14}C]$-tetranorbiotin (69). Lee et al. (85) reported the appearance of labeled bisnorbiotin and biotin sulfoxide in urine from rats intraperitoneally injected with $[^{14}C]$-biotin. Mock et al. (112) reported the appearance of $[^{14}C]$-bisnorbiotin, $[^{14}C]$-biotin sulfoxide and $[^{14}C]$-biotin sulfone in urine from pigs intravenously injected with $[^{14}C]$-biotin. Mock and Heird (105) observed that the urinary excretion of bisnorbiotin and biotin sulfoxide was substantially increased when humans were chronically supplemented with 1.2 mg of biotin per day. Whether oxidation of the thioether or $\beta$-oxidation is the major route of biotin catabolism is not clear. The concentration of bisnorbiotin is greater than that of biotin sulfoxide in urine and blood (Figure 2.7), suggesting $\beta$-oxidation is the major pathway of biotin degradation. However, that biotin sulfoxide is converted to biotin by a reductive system, such as biotin sulfoxide reductase (36) cannot be discarded.

Little is known about the factors that affect the degradation of biotin in vivo. Mock and Dyken (104) reported increased bisnorbiotin and biotin sulfoxide to biotin ratios in humans treated with anticonvulsants. Wang et al. (180) observed that the bisnorbiotin to biotin ratio in urine of rats was not affected by injection of anticonvulsants, but was increased by steroids. The oxidation of the sulfur atom when exposed to peroxides indicates that biotin is susceptible to oxidizing conditions. Similar to biotin, methionine has a thioether bond in its structure. Oxidation by reactive oxygen species converts methionine to methionine sulfoxide (118). Therefore, oxidative stress may affect the conversion
of biotin to biotin sulfoxide. Sealey et al. (160) reported that the ratio of biotin sulfoxide to biotin in urine is greater in smoking than in non-smoking humans.

The implications of understanding biotin degradation are multiple. For instance, understanding what drives biotin degradation would enhance our understanding on how and when biotin status of lactating dairy cows is affected, and therefore understand how biotin supplementation increases milk production. Additional information is also needed to understand biotin metabolism within the rumen. Santschi et al. (155) and Schwab et al. (158) attributed the decreased duodenal flow of biotin (relative to biotin intake) to microbial degradation. Because no information exists, determination of the conversion of labeled biotin into labeled biotin metabolites by rumen bacteria may confirm this postulate.

**Biotin and Foot Health in Farm Animals**

For several species, abnormalities in epidermal tissues, such as skin and hooves, were related to biotin deficiency (38,119). Cunha et al. (38) observed cracks in hooves of pigs consuming a basal diet containing 30% desiccated egg white. Pigs consuming the same basal diet that were injected with a daily dose of 100 μg of biotin intramuscularly did not manifest those signs. Mulling et al. (119) observed severe lesions in the skin and hooves of a calf to which biotin deficiency was induced by feeding egg white powder. Biotin supplementation had beneficial effects on foot health in pigs and horses that were not manifesting signs of clinical biotin deficiency (29,37,73,182). Webb et al. (182) reported that biotin supplementation improved hoof hardness in pigs. Comben et al. (37)
reported dramatic improvements in hoof hardness when three horses showing hoof defects (i.e., weak hooves) were supplemented with biotin. Buffa et al. (29) reported that biotin supplementation increased hoof growth rates in horses. Josseck et al. (73) observed that biotin supplementation improved hoof condition, but not hoof growth rate in horses.

Although incorrectly used (136), the term hoof health is frequently used to describe foot health. Whereas the equine foot consists of only one digit, the bovine foot consists of two digits that are known as lateral and medial claws. In addition to blood vessels, nerves, ligaments and tendons, each digit consists of the distal phalanx or pedal bone, the dermis or corium and the epidermis or epidermal hoof. Near the heel, the pedal bone is separated from the corium by a fourth structure, known as digital cushion. The hoof, which is continuous with the epidermis, encloses the corium. Contrary to the hoof, the corium is a highly vascular and innervated tissue (74). The vascular system consists of papillary arteries supplying blood to the capillary network of the dermis, which then is drained by papillary veins. Arteries and veins within the corium are also interconnected by arteriovenous anastomoses (74).

Keratinocytes are epidermal cells responsible for the synthesis of keratin. The stratum basale is a single layer of proliferating keratinocytes that lies over the corium (74,88). Keratins, also known as intermediate filaments, are a group of proteins expressed in epithelial cells (89). These proteins are found in tightly packed bundles, which are distributed throughout the cytoplasm. As epidermal cells differentiate, the cells condense and die, but the keratin filaments remain
intact forming a cell envelope (89). The stratum corneum is the outermost epidermal layer of keratinocytes, which are embedded in a lipid-rich extracellular matrix composed of cholesterol, fatty acids and ceramides.

The mechanisms by which biotin affects foot health are not very well understood. Keratin synthesis is a main determinant of foot health. Hendry et al. (64) showed less expression of keratin proteins (i.e., laminin and integrin) in the basement membrane for ulcerated bovine claws than for healthy bovine claws. Fritsche et al. (55) reported that synthesis of keratins was increased when keratinocytes were cultured in media with pharmacological biotin concentrations. Lischer et al. (88) observed that biotin supplementation improved the structure of the epidermis, although they did not report a direct measure of keratin synthesis. Healing time (i.e., time for solar hoof to cover the corium) in dairy cows manifesting sole ulcers was not affected by biotin supplementation (88). Keratin degradation can also impair hoof integrity (72,76). Kempson (76) reported the presence of the keratolytic bacteria *Bacteroides nodosum* in 27 horses with brittle hooves. Johnson et al. (72) reported greater collagenolytic activities for metalloproteinases in hoof tissue from horses with laminitis than in hoof tissue from healthy horses. Thiele et al. (173) observed increased keratin degradation when human skin was exposed to oxidative stress. Whether biotin has a protective effect against keratin degradation is unknown.

The lipid-rich matrix of the stratum corneum is a determinant of epidermal integrity. Higuchi et al. (65) observed that the concentrations of water and lipids in the sole horn of cows supplemented with biotin were lower and higher,
respectively, than those of control cows. Biotin is a cofactor for acetyl-CoA carboxylase, a key enzyme involved in fatty acid synthesis. Whether the beneficial effect of biotin on hoof integrity is related to increased fatty acid synthesis due to greater activity of acetyl-CoA carboxylase remains to be evaluated. In addition to cell to cell adhesion, the role of the lipid-rich matrix in maintaining epidermal integrity may also be related to controlling moisture content in epidermal cells. Choi et al. (35) reported that trans-epidermal water loss increased while lipid synthesis decreased when mice were subjected to insomniac psychology stress. The most common symptom of clinical biotin deficiency is dermatitis around the eyes, the mouth and the genital and perianal regions (113,161). Interestingly, these regions are typically exposed to moisture from tears, saliva, sweat and other secretions. Perhaps the control of water entry into the epidermis is also a major role of lipid-rich matrix. If this is correct, then the increased lipid content in sole horn (65) may explain how biotin supplementation enhances hoof integrity in farm animals.

The impairment of epidermal integrity may be related to decreased biotin uptake by epidermal cells. In human keratinocytes, the presence of a sodium-dependent transporter with very high affinity for biotin was reported (56). Different to SMVT, this biotin transporter is not inhibited by pantothenic acid or lipoic acid (56). The sole presence of this transporter would explain why the integrity of epidermal tissues is related to biotin status. The concentration of ABS in plasma of dairy cows ranges from 2.7 to 4.3 nmol/L (145,198). Given that this transporter (56) is saturable at physiological concentrations of biotin ($K_m = 2.6$ nmol/L), the
uptake of biotin into keratinocytes might be sensitive to biotin concentrations in blood. It is interesting, however, that another biotin transporter with the same characteristics of SMVT (i.e., sodium-dependent, saturable at a micromolar range and non-selective for pantothenic acid and lipoic acid) was detected in these keratinocytes (56). Grafe et al. (56) hypothesized that the SMVT has greater relevance for the transport of pantothenic acid than for the transport of biotin and, consequently, they justified the presence of a saturable transporter specific for biotin. It is also possible, however, that the SMVT transports biotin at high rates during optimal nutritional biotin status, but not during biotin deficiency. Pacheco-Alvarez et al. (128) observed that the gene expression of SMVT was down-regulated in liver and kidney cells when cultured in biotin-deficient media, although this effect was not observed in brain cells. These observations suggest that a prioritization exists for the transport of biotin into different tissues (128). Maybe the expression of SMVT in keratinocytes is rapidly down-regulated during biotin deficiency to spare biotin for other tissues, therefore, leaving the saturable transporter as the sole transporter for the uptake of biotin into keratinocytes.

**Biotin Regulation of Gene Expression**

The most notable function of biotin is its role as a cofactor of the biotin-dependent carboxylases. These enzymes play critical roles in lipid synthesis (acetyl-CoA carboxylase), gluconeogenesis (pyruvate carboxylase and propionyl-CoA carboxylase) and amino acid catabolism (methylcrotonyl-CoA carboxylase). In recent years, studies demonstrated that biotin also plays an important role in
the regulation of gene expression. Using DNA microarrays, Wiedmann et al. (187) evaluated the changes in gene expression in human PBMC after humans were supplemented with 8.8 μmol/d of biotin for 21 d, and reported that the expression of 139 genes increased and the expression of 131 genes decreased after biotin supplementation. Using the same technique, Rodriguez-Melendez et al. (143) evaluated the changes in gene expression in hepatocarcinoma cells, and reported that the expression of more than 2000 genes was increased or decreased in cells cultured at sub- or supra-physiological concentrations of biotin when compared to cells cultured at physiological concentrations. The exact mechanisms by which biotin regulates gene expression are still under evaluation, although evidence exists that biotin affects signal transduction and chromatin structure (194).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>Concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole egg¹</td>
<td>Microbiological assay</td>
<td>1.64</td>
<td>György and Rose (59)</td>
</tr>
<tr>
<td>Whole egg¹</td>
<td>Acid hydrolysis &amp; HPLC/avidin-binding assay</td>
<td>1.42</td>
<td>Staggs et al. (166)</td>
</tr>
<tr>
<td>Human milk²</td>
<td>Acid hydrolysis &amp; HPLC/avidin-binding assay</td>
<td>0.09</td>
<td>Mock et al. (108)</td>
</tr>
<tr>
<td>Cow milk²</td>
<td>Avidin-binding assay</td>
<td>0.21</td>
<td>Zimmerly and Weiss (198)</td>
</tr>
<tr>
<td>Green pea leaves³</td>
<td>Acid hydrolysis &amp; microbiological assay</td>
<td>0.28</td>
<td>Baldet et al. (10)</td>
</tr>
<tr>
<td>Pea seeds⁴</td>
<td>Avidin-binding assay</td>
<td>0.37</td>
<td>Duval et al. (45)</td>
</tr>
<tr>
<td>Barley grain</td>
<td>Acid hydrolysis &amp; microbiological assay</td>
<td>0.14</td>
<td>Abel et al. (2)</td>
</tr>
<tr>
<td>Hay</td>
<td>Acid hydrolysis &amp; microbiological assay</td>
<td>0.31</td>
<td>Abel et al. (2)</td>
</tr>
<tr>
<td>Swine diet</td>
<td>Acid hydrolysis &amp; microbiological assay</td>
<td>0.14</td>
<td>Bryant et al. (27)</td>
</tr>
<tr>
<td>Ruminant diet (45:55)</td>
<td>Acid hydrolysis &amp; microbiological assay</td>
<td>0.41</td>
<td>Midla et al. (98)</td>
</tr>
<tr>
<td>Ruminant diet (95:5)</td>
<td>Avidin-binding assay</td>
<td>0.18</td>
<td>Peterson et al. (132)</td>
</tr>
<tr>
<td>Ruminant diet (10:90)</td>
<td>Avidin-binding assay</td>
<td>0.10</td>
<td>Peterson et al. (132)</td>
</tr>
<tr>
<td>Ruminant diet (58:42)</td>
<td>Incubation with pancreatic extract &amp; avidin-binding assay</td>
<td>7.04</td>
<td>Santschi et al. (155)</td>
</tr>
</tbody>
</table>

¹ Assuming an egg weight (without shell) of 50 g and 15% DM concentration.
² Assuming 12% DM concentration.
³ Assuming 25% protein concentration (DM basis).
⁴ Values within parenthesis indicate the forage to concentrate ratio (F:C).

Table 2.1. Biotin concentration (mg/kg DM) of different foods, plant tissues and animal diets.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth Inhibition by Avidin</th>
<th>Inhibition Reversed by Biotin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>Complete</td>
<td>Reversed</td>
</tr>
<tr>
<td><em>Clostridium butyricum</em></td>
<td>Complete</td>
<td>Reversed</td>
</tr>
<tr>
<td><em>Clostridium chauvei</em></td>
<td>Complete</td>
<td>Reversed</td>
</tr>
<tr>
<td><em>Clostridium sporogenes</em></td>
<td>Complete</td>
<td>Reversed</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>Complete</td>
<td>Reversed</td>
</tr>
<tr>
<td><em>Lactobacillus arabinosus (L. plantarum)</em></td>
<td>Complete</td>
<td>Reversed</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>Complete</td>
<td>Reversed</td>
</tr>
<tr>
<td><em>Proteus morganii</em></td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td><em>Vibrio comma</em></td>
<td>None</td>
<td>-</td>
</tr>
</tbody>
</table>

Data from Landy et al. (83).

**Table 2.2.** Inhibition of growth by the inclusion of avidin in the media for the culture of gastrointestinal bacteria.
<table>
<thead>
<tr>
<th>Fluid</th>
<th>pH</th>
<th>([A^-]/[AH])</th>
<th>([A^-]), % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rumen</td>
<td>6.0</td>
<td>22</td>
<td>95.7</td>
</tr>
<tr>
<td>Stomach</td>
<td>2.0</td>
<td>2×10(^{-3})</td>
<td>0.2</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>7.0</td>
<td>224</td>
<td>99.6</td>
</tr>
</tbody>
</table>

\(^a\) Estimated using the Henderson-Hasselbalch equation: \(pH = pK_a + \log_{10} \left(\frac{[A^-]}{[HA]}\right)\).

**Table 2.3.** Form\(^a\) of biotin in typical body fluids based on its dissociation constant \((pK_a = 4.65)\).
Figure 2.1. Partial structure of diaminocarboxylic acid.

\[
\begin{align*}
C_8H_{13}S & \quad \left\{ \begin{array}{c}
- \text{COOH} \\
- \text{NH}_2 \\
- \text{NH}_2
\end{array} \right.
\end{align*}
\]

Figure 2.2. Conversion of diaminocarboxylic acid into a cyclic urea derivative.

\[
\begin{align*}
C_8H_{13}S & \quad \left\{ \begin{array}{c}
- \text{COOH} \\
- \text{NH}_2 \\
- \text{NH}_2
\end{array} \right. & \xrightarrow{\text{Cl}_2\text{CO}} & \quad C_8H_{13}S & \quad \left\{ \begin{array}{c}
- \text{COOH} \\
- \text{NH}_2 \\
- \text{NH}_2 \end{array} \right. \\
& & & \quad \left\{ \begin{array}{c}
\text{NH} \quad \text{C}=\text{O}
\end{array} \right.
\end{align*}
\]

Figure 2.3. (A) When subjected to H\textsubscript{2}O\textsubscript{2}, diaminocarboxylic acid is converted into a sulfone, indicating the presence of a thioether bond in its structure. (B) Partial structure of diaminocarboxylic acid.
Figure 2.4. Biotin contains a 5-membered urea ring structure, also known as imidazolidone or ureido ring.

Figure 2.5. Structure of adipic acid.

Figure 2.6. Structure of biotin.
Figure 2.7. Percentage of the total avidin-binding activity in urine (A), serum (B) and milk (C) from humans as determined by a two-step procedure for the quantification of biotin and biotin analogues. Data from Mock et al. (109-111).
Figure 2.8. Pathway of biotin synthesis.
Figure 2.9. Biotin intake (solid bars), biotin balance (open bars) and DM intake (triangles) in ewes consuming diets containing different forage to concentrate ratios (F:C). Biotin balance was estimated as biotin output in urine and feces minus biotin intake. Data from Peterson et al. (132).
3-HYDROXY-ISOVALERIC ACID IN URINE IS NOT A SENSITIVE INDICATOR OF BIOTIN STATUS IN LACTATING DAIRY COWS

ABSTRACT

A sensitive indicator of biotin status for lactating dairy cows is necessary to understand the factors that affect the response of milk yield to biotin supplementation. 3-hydroxy-isovaleric acid (3HIA) is an alternative metabolite in the pathway of leucine catabolism when the biotin-dependent enzyme methylcrotonyl-CoA carboxylase is limiting. We evaluated the urinary excretion of 3HIA as a determinant of biotin status. We hypothesized that high-producing cows have a greater biotin requirement, and therefore would excrete more 3HIA than low-producing cows. We also hypothesized that biotin supplementation decreases 3HIA excretion. Twenty high-producing and 20 low-producing Holstein cows (43±5 and 23±4 kg/d of milk yield, respectively) were fed a basal diet that contained 0 or 0.96 mg/kg of supplemental biotin. On d 16 cows were given an intraruminal infusion of 1.4 mol of isovaleric acid (IVA). Urine samples were
collected at 0, 8, 12, 24 and 48 h after infusion. Biotin supplementation increased milk yield in high-producing cows, but not in low-producing cows. Neither production nor biotin supplementation affected the basal urinary excretion of 3HIA. Infusion with IVA substantially increased urinary excretion of 3HIA, which was greatest 8 h after infusion. Biotin supplementation did not attenuate this increase. The increase in urinary 3HIA excretion was less for low-producing cows than for high-producing cows. We concluded that urinary excretion of 3HIA is not a sensitive determinant of biotin status. A sensitive indicator of biotin status for lactating dairy cows is still needed to understand which factors affect the response to biotin supplementation.

INTRODUCTION

Biotin requirements for lactating dairy cows have not been established (122). Because of its supply by feeds and synthesis by rumen microorganisms (99,199), classical biotin deficiency (i.e., clinical deficiency) is unlikely to occur in adult or functioning ruminants. Despite this, several studies (90,98,198), but not all (51,145), showed that biotin supplementation increases milk yield by lactating dairy cows. The response in milk yield might be affected by several factors (e.g., diet composition, lactation stage, and others), but also may be dependent on whether the biotin status of lactating dairy cows is optimum to maximize milk yield. To improve our understanding of the effects of biotin supplementation on
milk yield, a sensitive indicator of biotin status for lactating dairy cows is necessary.

Concentrations of avidin-binding substances (ABS) in blood and milk have been used typically as measures of biotin status in lactating dairy cows (145,198). In addition to biotin, ABS include biotin metabolites, such as bisnorbiotin and biotin sulfoxide, which may lack vitamin activity (96). In humans, biotin accounts for approximately 50 and 70% of the total ABS in serum (110) and milk (111), respectively. These data suggest that concentration of ABS in plasma and milk may not be adequate indicators of biotin status for lactating dairy cows.

Mock et al. (117) evaluated different determinants of biotin status in humans and concluded that urinary excretion of 3-hydroxy-isovaleric acid (3HIA) is an early and sensitive indicator of marginal biotin deficiency. 3-hydroxy-isovaleric acid is the alternative metabolite formed from 3-methylcrotonyl-CoA when the activity of a biotin-dependent enzyme, methylcrotonyl-CoA carboxylase (MCC), is limiting (170). Under the assumption that the activity of MCC is limiting in dairy cows, we hypothesized that the urinary excretion of 3HIA decreases when biotin is supplemented. We also hypothesized that high-producing cows have a greater need for biotin than low-producing cows and consequently, urinary excretion of 3HIA is greater for high-producing than for low-producing cows.

The objectives of this study were: 1) to evaluate the urinary excretion of 3HIA as a determinant of biotin status for lactating dairy cows; and 2) to
determine whether the response in milk yield to biotin supplementation is dependent on milk production.

**MATERIALS AND METHODS**

**Animals, Diets and Challenge**

All procedures involving animals were approved by the Agricultural Animal Care and Use Committee of The Ohio State University. Twenty multiparous high-producing (43±5 kg/d of milk yield and 136±56 days after parturition at the beginning of the experiment) and twenty multiparous low-producing Holstein cows (23±4 kg/d of milk yield and 267±53 days after parturition at the beginning of the experiment) were used. Cows within the same production group were blocked by milk yield (average of the 7-d period previous to the beginning of the experiment) and randomly assigned to one of two treatments. Treatments consisted of a diet (Table 3.1) that contained 0 or 0.96 mg of supplemental biotin per kg of dry matter (DM). The concentration of supplemental biotin was selected to supply 20 mg per day (90,98,198) based on an estimated DM intake of 22 kg/d. The diet was formulated to meet nutrient requirements (122) for a 650-kg lactating dairy cow producing 40 kg of milk per day. High proportions of grain in the substrate decreased microbial biotin synthesis in vitro (2); therefore, we formulated the basal diet to contain a high proportion of concentrate (60% DM basis). Corn gluten meal (~75% ruminally undegraded protein, 122) was the main protein source because it has high concentrations of leucine (122), the substrate
for MCC. Supplemental biotin (Rovimix® Biotin, DSM Nutritional Products Inc., Parsippany, NJ) was supplied in the pelleted concentrate mix. Cows were housed in individual tie-stalls and fed once daily (0400 h). Diets were offered ad libitum (~5% feed refusals, as-fed basis) as mixed rations. Amount of feed offered and refused was measured daily. Diets were adjusted weekly for changes in ingredient DM concentration. Cows were milked twice daily (0200 and 1300 h) and milk weights were recorded electronically at each milking. The average from the 14 daily milk yields was used as the value for milk yield. Cows were weighed on d 14 at 0800 h.

On d 16, cows were given a pulse intraruminal dose of 1.4 mol of IVA. The solution of IVA was prepared on d 15 by mixing 150 mL of isovaleric acid (TCI America, Inc., Portland, OR) with 3.5 L of distilled water. The solution was adjusted to pH = 6.2 by adding ~50 g of NaOH. Intraruminal infusions were done at 0800 h by stomach tubing and took approximately 2 min per cow.

Sample Collection and Analysis

Samples of feeds and feed refused were collected once weekly. An aliquot of feed and feed refusal samples were dried in a forced-air oven for 24 h at 100°C. Dry matter intake was measured as the difference between DM offered and DM refused. A second aliquot of feed was stored at -20°C until analysis. Forage samples were lyophilized and all feed samples were ground to pass through a 1-mm screen of a Wiley mill (Arthur A. Thomas, Philadelphia, PA). Ground samples were analyzed for DM (24 h at 100°C), neutral detergent fiber
(Ankom™ Fiber Analyzer, Ankom Technology, Fairport, NY) with sodium sulfite and α-amylase (Ankom Technology), crude protein (Kjeldahl N × 6.25), ash (6), starch (185) and long-chain fatty acids (168).

Samples of rumen fluid were collected on d 15 via stomach tube. Rumen fluid was processed and analyzed for volatile fatty acid concentration as described by Zimmerly and Weiss (198).

Milk samples (a.m. and p.m. milkings) were collected on d 7 and 14 for determination of milk fat, protein and lactose concentrations with a B2000 Infrared Analyzer (Bentley Instruments, Chaska, MN) by DHI Cooperative, Inc. (Columbus, OH). Milk yields from d 7 and 14 were used to calculate yields of milk components and energy-corrected milk (ECM) yield. Energy-corrected milk (kg/d) yield was calculated as ECM = \[41.62 \times F + 24.16 \times P + 21.60 \times L - 11.72\] \(\div\) 340, where F, P and L are concentrations (g/kg) of fat, protein and lactose in milk and MY (kg/d) is milk yield (176).

An additional milk sample was collected on d 15 (p.m. milking) for determination of ABS concentration. This sample was stored at 4°C immediately after collection. Within four h after collection, milk samples were warmed in a water-bath at 39°C for 10 min and homogenized by repeated (5X) pouring into a beaker. A 10-mL aliquot of homogenized whole milk was centrifuged at 20,000×g for 20 min at 4°C. After removing the fat layer, skim milk was decanted and stored at -20°C until ABS analysis. Concentration of ABS in skim milk (1:400 and 1:800 dilution for Control and Biotin, respectively) was determined by a single-step competitive enzyme binding assay (Ridascreen® Biotin kit, R-Biopharm
GmbH, Germany). Concentrations of ABS are reported on a whole-milk basis, which were calculated as the concentration of ABS in skim milk (nmol/L) multiplied by the ratio of skim-milk to whole-milk.

Blood samples were collected at 0800 h of d 15 by venipuncture of the coccygeal vein (18-G needle), immediately transferred into heparinized tubes, stored in ice and transferred to the laboratory. Blood plasma was obtained after centrifugation of blood at 4,000×g for 30 min and stored at -20°C until ABS analysis. Concentrations of ABS in plasma (1:10 dilution) were determined as described above.

Urine samples (~300 mL) were collected by vulva stimulation at 0, 8, 12, 24 and 48 h post-infusion. Urine samples (20 mL) were centrifuged at 4,000×g for 20 min, decanted and stored at 4°C until analysis for 3HIA and creatinine concentrations. All samples were analyzed within one week of collection. Urine samples were extracted as described by Truscott et al. (175) with one additional acidic extraction with ethyl acetate (107). To facilitate the separation of phases, each extraction was performed after a 3-min centrifugation at 20,000×g. A 1-mL aliquot of the extract was dried at room temperature under N₂ stream. The dried extract was solubilized and derivatized as described by Mock et al. (115) using 50 μL of pyridine and 50 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 1% trimethylchlorosilane (TMCS) (Alltech Associates, Inc., Deerfield, IL). GLC/MS analysis was performed using an HP 6890 GC (Agilent Technologies Inc., Palo Alto, CA) with a 30 m × 0.25 mm HP-5MS capillary column (Agilent Technologies Inc.) connected to an HP 5973 Mass Selective Detector (Agilent Technologies...
Inc.). The oven temperature was programmed as described by Mock et al. (114). For quantification, 3HIA (55453 Fluka, Sigma Diagnostics, St. Louis, MO) was used as an external standard. To improve resolution, the MS detector was set in the selected ion-monitoring (SIM) mode to detect the molecular-ion peak at m/z 131. The recovery of 3HIA from urine after extraction was 96%. The intra assay CV for 3HIA measurement was 6.2%. Creatinine concentration in urine was determined by the method of Jaffe (39). Excretion of 3HIA was expressed as mmol of 3HIA per mol of creatinine. Concentrations of ABS in urine (1:1000 dilution) were determined as described above. Daily ABS excretion in urine was calculated using the estimated creatinine excretion. Creatinine excretion (mg/d) was calculated as $29 \times \text{BW (kg)}$, where BW is the body weight (177).

**Statistical Analyses**

Ten cows per treatment were used in this study. One low- and one high-producing cow that were assigned to the control and biotin diets, respectively, were removed from the experiment due to health problems (right-displaced abomasum and undefined infection, respectively). Data from these cows were not used for statistical analyses.

Production measures, ABS concentrations in fluids, and baseline urinary excretion of 3HIA were analyzed as a randomized complete block design using the MIXED procedure of SAS (version 9.1, SAS Institute, Inc., Cary, NC). The model included the effects of block (random, df = 9), diet (fixed, df = 1), production (fixed, df = 1), the interaction of diet by production (fixed, df = 1) and
the residual error (random, df = 25). The effect of IVA challenge on 3HIA excretion was analyzed as a randomized complete block design with repeated measures using the MIXED procedure of SAS. The model included the effects of block (random, df = 9), diet (fixed, df = 1), production (fixed, df = 1), the interactions of diet by production (fixed, df = 1), the whole plot error (random, df = 25), the effect of time (fixed, df = 4), the interactions of time by diet (fixed, df = 4), time by production (fixed, df = 4), time by diet by production (fixed, df = 4), and the residual error (random, df = 139). The heterogeneous autoregressive covariance structure was selected for analysis with repeated measures based on the Akaike’s information criterion. Significant difference between main effects was declared at P < 0.05, whereas significant interaction of main effects was declared at P < 0.10. If a significant interaction was observed the pdiff option was used to test differences among treatments.

RESULTS

Production Performance

High-producing cows consumed more DM than low-producing cows (P < 0.01), but biotin supplementation had no effect on DM intake (Table 3.2). A production by biotin supplementation interaction was observed for milk yield (P < 0.09) and ECM yield (P < 0.07). Biotin supplementation increased milk yield (P < 0.01) and ECM yield (P < 0.01) in high-producing cows, but not in low-producing cows. Biotin supplementation did not affect the concentrations of fat, protein and
lactose in milk. A production by biotin supplementation interaction was significant for fat yield ($P < 0.09$). Biotin supplementation increased fat ($P < 0.01$) yields in high-producing cows, but not in low-producing cows. Biotin supplementation increased lactose yield ($P < 0.03$), but did not affect protein yield.

**Volatile Fatty Acids in Rumen Fluid**

High-producing cows had lesser ($P < 0.04$) and greater ($P < 0.05$) molar proportions of acetate and propionate in rumen fluid, respectively, than low-producing cows (Table 3.3). The acetate to propionate ratio in rumen fluid was less for high-producing cows than for low-producing cows ($P < 0.01$). Biotin supplementation did not affect the molar proportions of acetate, propionate, butyrate, isobutyrate and isovalerate, or the acetate to propionate ratio in rumen fluid. A production by biotin supplementation interaction was observed for the molar proportion of valerate ($P < 0.07$).

**Biotin Status**

The concentrations of ABS in plasma and milk were not affected by production (Table 3.4), but were increased by biotin supplementation in both high-producing and low-producing cows ($P < 0.01$). Biotin supplementation increased the output of ABS in milk ($P < 0.01$) and this increase was greater for high-producing than for low-producing cows ($P < 0.01$). The ABS to creatinine ratio in urine was not affected by production, but was increased by biotin supplementation ($P < 0.01$). The output of ABS in urine was increased by biotin
supplementation, and tended to be greater for low-producing than for highproducing cows ($P < 0.09$). Neither production nor biotin supplementation affected the basal (i.e., pre-infusion) urinary excretion of 3HIA (Table 3.4). Infusing 1.4 mol of IVA substantially increased the urinary excretion of 3HIA ($P < 0.01$), which was greatest 8 h after infusion (Figure 3.1). Biotin supplementation did not attenuate this increase. The increase in urinary 3HIA excretion was less for low-producing cows than for high-producing cows ($P < 0.01$).

**DISCUSSION**

We hypothesized that high-producing cows have a greater demand for biotin than low-producing cows. In agreement with our expectations, biotin supplementation increased milk and ECM yields in high-producing cows (5.4 and 7.8 kg/d, respectively), but had no effect on these variables in low-producing cows. This interaction agrees with results from most studies using low-producing (51) and high-producing cows (90,98,198). Reasons for the lack of response to biotin supplementation by high-producing cows ($\geq 35$ kg/d) in another study (145) are unclear. The milk yield increase in this study was much greater (13% vs. 2.5 to 7.5%) than that observed in other studies (90,98,198). Our study only lasted 14 d and was not designed primarily to evaluate responses in milk yields. Therefore, the magnitude of the response should be interpreted with caution.

The mechanism by which biotin supplementation increases milk yield is still unknown. Because certain cellulolytic bacteria require biotin for growth (159),
increased growth of cellulolytic bacteria in response to biotin supplementation could possibly have increased DM digestibility and, consequently, milk yield. However, Majee et al. (90) reported that biotin supplementation did not affect DM or fiber total tract digestibility in high-producing dairy cows. Also, an increase in the population of cellulolytic bacteria would likely increase the molar proportions of acetate in rumen fluid. Molar proportions of acetate and propionate were affected by production level, but not by biotin supplementation (Table 3.3). This last observation is consistent with previous observations (198).

Biotin supplementation increased lactose yield (Table 3.2). This observation is consistent with data of Majee et al. (90). Unfortunately, neither Zimmerly and Weiss (194) nor Rosendo et al. (145) reported milk lactose concentrations or yields. Because lactose secretion is the main determinant of milk volume, these observations suggest that the response in milk yield to biotin supplementation may be indirectly related to lactose secretion, presumably by an increased supply of glucose to the mammary gland. Zimmerly and Weiss (194) hypothesized that glucose production could be increased by biotin supplementation. Pyruvate carboxylase and propionyl-CoA carboxylase are two biotin-dependent enzymes involved in gluconeogenesis, and biotin may have increased the activity of these enzymes in the liver of high-producing dairy cows.

Biotin supplementation increased fat yield by high-producing cows (Table 3.2). This increase may also be indirectly related to an increase of gluconeogenesis in the liver. Increased glucose supply to the mammary gland may increase glucose oxidation through the pentose phosphate pathway (123),
thereby increasing the availability of reducing equivalents for fatty acid synthesis. Another explanation for the increased fat yield in high-producing cows may be related to a direct effect of biotin supplementation on fatty acid synthesis by increasing the activity of acetyl-CoA carboxylase. Zimmerly and Weiss (198) and Rosendo et al. (145) did not observe an effect of biotin supplementation on fat yield by lactating dairy cows in early lactation (less than 100 days after parturition). However, the lack of response in fat yield to biotin supplementation in these studies may be related to the inhibition of de novo synthesis of fatty acids due to the negative energy balance observed during early lactation (129). Majee et al. (90) did not observe an increase in fat yield when high-producing cows in mid lactation were supplemented with biotin.

Concentrations of ABS in blood and milk are typically used as a measure of biotin status for lactating dairy cows (145,198). The concentrations of ABS in milk and plasma were highly and positively correlated ($r = 0.90$, $P < 0.01$, Figure 3.2), suggesting that changes in biotin intake would affect these measures similarly. Under the hypothesis that high-producing cows have a greater demand for biotin than low-producing cows, we expected lower concentrations of ABS in plasma, milk and urine for high-producing cows than for low-producing cows. Contrary to our expectations, high-producing and low-producing cows consuming the control diet had similar ABS concentrations in plasma and milk and similar ABS to creatinine ratios in urine (Table 3.4). Milk production responses to biotin supplementation, however, differed between production groups. Based on this, our data suggest that ABS concentrations in plasma and milk and ABS to
creatine ratios in urine are not sensitive determinants of biotin status. Because we did not use a two-step procedure (110,111), ABS concentrations may not approximate actual biotin concentrations. However, data from humans also showed that the concentration of actual biotin in blood is not a sensitive determinant of biotin status (117).

Isovaleric acid, in the form of isovaleryl-CoA, is an intermediate metabolite from the catabolism of leucine (170). The biotin-dependent enzyme MCC is required in one intermediary step of the pathway for the complete catabolism of leucine or IVA. In the presence of MCC, 3-methylcrotonyl-CoA is further metabolized to acetoacetate and acetyl-CoA. However, when the activity of MCC is limiting, 3-methylcrotonyl-CoA is alternatively metabolized to 3HIA by the action of the enzyme enoyl hydratase or crotonase (114,170,172). The resulting 3HIA is excreted in urine (81,171,172). Under the assumption that MCC activity in lactating dairy cows is limiting, we expected greater excretions of 3HIA in high-producing than in low-producing cows. High-producing and low-producing cows consuming the control diet had similar basal urinary excretions of 3HIA (Table 3.4). Also, urinary excretion of 3HIA did not decrease when biotin was supplemented. One explanation for these observations is that 3HIA could have been formed directly from 2-ketoisocaproic acid by the action of ketoisocaprate oxygenase (86,147). Even though this possibility cannot be discarded, this biotin-independent pathway implies a wasting of energy given that IVA would be excreted without being further metabolized. Another explanation for the similar excretion of 3HIA among treatments is that the activity of MCC was adequate in
these cows and, therefore the substrate load was inferior to the maximum capacity of MCC. To overload the capacity of MCC, cows were given an intraruminal infusion with 1.4 moles of IVA. This dose, which is equivalent to 2.8 times the mean daily absorption of IVA (3-methyl isomer) estimated from net flux from portal-drained viscera in lactating dairy cows (15,31,140), was sufficient to substantially increase urinary 3HIA excretion in all treatments (Figure 3.1). This observation suggests either that the capacity of MCC was overwhelmed or that IVA was converted to 3HIA by alternative pathways (86,147). Even though it could be involved in the conversion of 3HIA from endogenous sources (i.e., leucine catabolism), 2-ketoisocaproate oxygenase would probably not be involved in the conversion of 3HIA from exogenous sources such as infused IVA. Isovaleryl-CoA is the product of the oxidative decarboxylation of 2-ketoisocaproic acid by branched-chain amino acid dehydrogenase. Because this is an irreversible reaction (20), exogenous IVA would probably not be converted into 3HIA through this pathway. Another alternative pathway would be the direct hydroxylation of exogenous IVA to 3HIA by isovaleric acid hydroxylase (147). There is no information regarding isovaleric acid hydroxylase in ruminants, but if this alternative biotin-independent pathway is present, urinary 3HIA excretion may not be a valid determinant of biotin status.

Under the assumption that MCC activity is limiting, we expected biotin supplementation to attenuate the increase in urinary 3HIA excretion after the IVA challenge. We also expected this attenuation to be greater for high-producing than for low-producing cows. Contrary to our expectations, the increase in urinary
3HIA excretion was similar for supplemented and non-supplemented cows. Although this was observed for both groups of cows, the increase in urinary 3HIA was greater for high-producing than for low-producing cows (Figure 3.1) suggesting that the substrate load of IVA was closer to maximum MCC capacity in high-producing than in low-producing cows. Greater loads of IVA for high-producing than for low-producing cows may be attributed to a greater DM intake (Table 3.2), a change in ruminal fermentation (Table 3.3) or a combination of both. It is tempting to suggest that a similar activity of MCC for non-supplemented and supplemented cows explains the similar increase in the excretion of 3HIA. However, the possibility of the direct conversion of IVA to 3HIA by isovaleric acid hydroxylase should not be discarded. No matter which pathway, urinary 3HIA excretion was not a sensitive determinant of biotin status for lactating dairy cows under these experimental conditions.

Contrary to lactating dairy cows, urinary excretion of 3HIA is a sensitive determinant of biotin status in non-ruminants, such as humans (117) and rats (116,172). This difference may be explained from an evolutionary perspective. Isovaleric acid (an end-product of bacterial fermentation) is absorbed by the gastrointestinal tract and then released into the portal vein. Daily net release of isovaleric acid (both isomers) by portal drained viscera for growing pigs (137,193) and lactating dairy cows (140) was approximately 1.3 and 14.9 mmol per kg of metabolic body weight, respectively. Lactating dairy cows, and likely other ruminants, must have evolved to metabolize increased loads of IVA resulting from bacterial fermentation in the rumen.
In conclusion, biotin supplementation increased milk production in high-producing cows but not in low-producing cows. The mechanism by which biotin increases milk production is still unclear. The typical measures of biotin status, such as ABS concentration in plasma, milk or urine or 3HIA excretion in urine, are not sensitive determinants of biotin status for lactating dairy cows. A sensitive indicator of biotin status for lactating dairy cows is still needed to understand the factors that affect the response in milk yield to biotin supplementation by lactating dairy cows.

ACKNOWLEDGMENTS

Appreciation is extended to Mr. Kevin Miller and the crew of the Krauss Dairy Center at the OARDC for the day-to-day cow management and for their assistance with rumen infusions. Gratitude is extended to Mrs. Donna Wyatt and Mrs. Dianne Borger for their assistance in the laboratory.
### Table 3.1. Ingredients $^1$,$^2$ and nutrient composition (% DM basis) of the basal diet.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition (%)</th>
</tr>
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<tbody>
<tr>
<td>Corn silage</td>
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<tr>
<td>Alfalfa silage</td>
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</tr>
<tr>
<td>Corn grain (dry and ground)</td>
<td>21.9</td>
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<tr>
<td>Corn gluten meal</td>
<td>10.0</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>16.9</td>
</tr>
<tr>
<td>Soybean meal (44% crude protein)</td>
<td>8.0</td>
</tr>
<tr>
<td>Fat (animal and vegetable blend)</td>
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</tr>
<tr>
<td>Urea</td>
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</tr>
<tr>
<td>Sodium bicarbonate</td>
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</tr>
<tr>
<td>Minerals and vitamins</td>
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</tr>
<tr>
<td><strong>Chemical analysis</strong></td>
<td></td>
</tr>
<tr>
<td>Crude protein</td>
<td>20.7</td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
<td>33.0</td>
</tr>
<tr>
<td>Starch</td>
<td>24.6</td>
</tr>
<tr>
<td>Long-chain fatty acids</td>
<td>4.0</td>
</tr>
<tr>
<td>Ash</td>
<td>5.5</td>
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</table>

$^1$ Diet with supplemental biotin contained 0.0048% of biotin premix (Rovimix® H-2, 20 mg/g), which replaced a similar amount of corn gluten meal.

$^2$ The diet contained 2.0 g/kg dicalcium phosphate, 3.6 g/kg limestone, 0.1 g/kg magnesium oxide, 2.1 g/kg trace mineral salt, 0.32 mg/kg selenium (200 mg/kg), 0.024 g/kg zinc sulfate, 0.012 g/kg copper sulfate, 3,300 IU/kg vitamin A, 810 IU/kg vitamin D, and 22 IU/kg vitamin E.
<table>
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<tr>
<th></th>
<th>Low-producing</th>
<th>High-producing</th>
<th>SEM</th>
<th>$P$-value</th>
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<tr>
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<td>Biotin</td>
<td>Control</td>
<td>Biotin</td>
<td>Biotin</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>746</td>
<td>743</td>
<td>660</td>
<td>657</td>
<td>22</td>
</tr>
<tr>
<td>DM intake, kg/d</td>
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<td>21.1</td>
<td>23.9</td>
<td>25.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Milk yield, kg/d</td>
<td>23.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4</td>
</tr>
<tr>
<td>Energy-corrected milk yield, kg/d</td>
<td>22.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8</td>
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<td>Fat concentration, %</td>
<td>3.70</td>
<td>3.79</td>
<td>3.47</td>
<td>3.95</td>
<td>0.20</td>
</tr>
<tr>
<td>Fat yield, kg/d</td>
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<td>0.94&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09</td>
</tr>
<tr>
<td>True protein concentration, %</td>
<td>3.44</td>
<td>3.34</td>
<td>2.95</td>
<td>2.93</td>
<td>0.09</td>
</tr>
<tr>
<td>True protein yield, kg/d</td>
<td>0.80</td>
<td>0.82</td>
<td>1.18</td>
<td>1.34</td>
<td>0.05</td>
</tr>
<tr>
<td>Lactose concentration, %</td>
<td>4.76</td>
<td>4.76</td>
<td>4.81</td>
<td>4.90</td>
<td>0.08</td>
</tr>
<tr>
<td>Lactose yield, kg/d</td>
<td>1.11</td>
<td>1.17</td>
<td>1.93</td>
<td>2.25</td>
<td>0.08</td>
</tr>
</tbody>
</table>

$^1$ $P =$ effect of production; $B =$ effect of biotin supplementation; $P \times B =$ interaction of production by biotin supplementation.

$^{a,b,c}$ Numbers with different superscripts differ ($P < 0.05$).

**Table 3.2.** Body weight, dry matter (DM) intake and production of low-producing and high-producing dairy cows consuming a diet containing 0 (Control) or 0.96 mg/kg DM (Biotin) of supplemental biotin.
<table>
<thead>
<tr>
<th></th>
<th>Low-producing Control</th>
<th>Biotin</th>
<th>High-producing Control</th>
<th>Biotin</th>
<th>SEM</th>
<th>P-value</th>
<th>B</th>
<th>P×B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate, mol/100 mol VFA</td>
<td>61.1</td>
<td>62.5</td>
<td>58.6</td>
<td>58.3</td>
<td>1.0</td>
<td>0.01</td>
<td>0.59</td>
<td>0.42</td>
</tr>
<tr>
<td>Propionate, mol/100 mol VFA</td>
<td>21.3</td>
<td>20.3</td>
<td>24.5</td>
<td>24.7</td>
<td>1.2</td>
<td>0.01</td>
<td>0.73</td>
<td>0.60</td>
</tr>
<tr>
<td>Butyrate, mol/100 mol VFA</td>
<td>13.3</td>
<td>13.2</td>
<td>12.7</td>
<td>13.0</td>
<td>0.6</td>
<td>0.57</td>
<td>0.90</td>
<td>0.75</td>
</tr>
<tr>
<td>Isobutyrate, mol/100 mol VFA</td>
<td>0.8</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
<td>0.1</td>
<td>0.29</td>
<td>0.91</td>
<td>0.32</td>
</tr>
<tr>
<td>Valerate, mol/100 mol VFA</td>
<td>2.3</td>
<td>2.0</td>
<td>2.2</td>
<td>2.2</td>
<td>0.2</td>
<td>0.65</td>
<td>0.16</td>
<td>0.07</td>
</tr>
<tr>
<td>Isovalerate, mol/100 mol VFA</td>
<td>1.2</td>
<td>1.2</td>
<td>1.3</td>
<td>1.1</td>
<td>0.1</td>
<td>0.85</td>
<td>0.44</td>
<td>0.65</td>
</tr>
<tr>
<td>Acetate:Propionate</td>
<td>2.9</td>
<td>3.1</td>
<td>2.4</td>
<td>2.5</td>
<td>0.2</td>
<td>0.01</td>
<td>0.50</td>
<td>0.64</td>
</tr>
</tbody>
</table>

1° P = effect of production; B = effect of biotin supplementation; P × B = interaction of production by biotin supplementation.

Table 3.3. Molar proportions of volatile fatty acids (VFA) in rumen fluid from low-producing and high-producing dairy cows consuming a diet containing 0 (Control) or 0.96 mg/kg DM (Biotin) of supplemental biotin.
<table>
<thead>
<tr>
<th></th>
<th>Low-producing</th>
<th>High-producing</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Biotin</td>
<td></td>
</tr>
<tr>
<td>Plasma ABS, nmol/L</td>
<td>4.3</td>
<td>7.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Milk ABS, nmol/L</td>
<td>141</td>
<td>472</td>
<td>31</td>
</tr>
<tr>
<td>Milk ABS output, µmol/d</td>
<td>3.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.2</td>
</tr>
<tr>
<td>Urine ABS, µmol/mol creatinine</td>
<td>95</td>
<td>201</td>
<td>13</td>
</tr>
<tr>
<td>Urine ABS output,&lt;sup&gt;2&lt;/sup&gt; µmol/d</td>
<td>8.9</td>
<td>19.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Milk + urine ABS output, µmol/d</td>
<td>12.1</td>
<td>30.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Urine 3HIA, mmol/mol creatinine</td>
<td>66.0</td>
<td>70.0</td>
<td>9.7</td>
</tr>
</tbody>
</table>

<sup>1</sup> P = effect of production; B = effect of biotin supplementation; P × B = interaction of production by biotin supplementation.

<sup>2</sup> Estimated assuming a creatinine excretion of 29 mg per kg of body weight (177).

**Table 3.4.** Concentration of avidin-binding substances (ABS) in plasma, milk and urine, output of ABS in milk and urine, and basal (i.e., pre-infusion) urinary concentration of 3-hydroxyisovaleric acid (3HIA) of low-producing and high-producing dairy cows consuming a diet containing 0 (Control) or 0.96 mg/kg DM (Biotin) of supplemental biotin.
Figure 3.1. Urinary excretion of 3-hydroxy-isovaleric acid (3HIA) of lactating dairy cows after an intraruminal infusion (d 16) of 1.4 mol of isovaleric acid (3-methyl isomer). High-producing (HP) and low-producing (LP) cows were fed a diet containing 0 (Control) or 0.96 mg/kg DM (Biotin) of supplemental biotin. Values are means ± SEM.
Figure 3.2. Relationship between the concentrations of avidin-binding substances (ABS) in milk and plasma of lactating dairy cows.
CHAPTER 4

EFFECT OF BIOTIN SUPPLEMENTATION ON ENZYME ACTIVITY AND GENE EXPRESSION OF BIOTIN-DEPENDENT CARBOXYLASES IN THE LIVER OF DAIRY COWS

ABSTRACT

The mechanism by which biotin supplementation increases milk production is not known. Biotin is a cofactor of the gluconeogenic enzymes, propionyl-CoA carboxylase (PCC) and pyruvate carboxylase (PC). We hypothesized that biotin supplementation increases the activity and the gene expression of PCC and PC, and the gene expression of phosphoenolpyruvate carboxykinase (PEPCK) in the liver of high producing dairy cows. Eight multiparous Holstein cows (40±2 kg/d milk yield and 162±35 days after parturition) were randomly assigned to one of two diet sequences according to a cross-over design with two 22-d periods. Treatments consisted of a basal diet (60% concentrate) containing 0 or 0.96 mg/kg of supplemental biotin. On d 21 from each period, liver tissue was collected by percutaneous liver biopsy.
Activities of PCC and PC were determined by measuring the fixation of $[^{14}\text{C}]\text{O}_2$ in liver homogenates. Abundance of mRNA for PCC, PC and PEPCK was determined by quantitative RT-PCR. Biotin supplementation tended ($P < 0.12$) to increase the activity of PC (11 vs. 15 nmol CO$_2$.min$^{-1}$.mg protein$^{-1}$), but did not affect the activity of PCC (20 nmol CO$_2$.min$^{-1}$.mg protein$^{-1}$). The differential response to biotin supplementation suggests that PCC may have a higher priority for biotin than PC. Biotin supplementation did not affect the gene expression of PCC, PC and PEPCK. We attributed the increased activity of PC without changes in mRNA abundance to an increased activation of the apoenzymes by holocarboxylase synthetase. In conclusion, biotin supplementation can affect the activity of biotin-dependent carboxylases in the liver of lactating dairy cows. Whether biotin supplementation increases glucose production in the liver still needs to be evaluated.

**INTRODUCTION**

Several studies demonstrated that biotin supplementation increases milk production by lactating dairy cows (90, 98, 198). Despite these observations, the mechanism by which biotin supplementation increases milk production is still not known. Glucose supply to mammary gland is a major determinant of milk yield in lactating dairy cows (139). Therefore, the increase in milk production by lactating dairy cows supplemented with biotin might be related to an increase in gluconeogenesis, presumably in the liver (138).
Biotin is a cofactor of the gluconeogenic enzymes, pyruvate carboxylase (PC) and propionyl-CoA carboxylase (PCC). Pyruvate carboxylase catalyzes the carboxylation of pyruvate to form oxaloacetate (75), whereas PCC catalyzes the carboxylation of propionyl-CoA to form methylmalonyl-CoA (52), a precursor of oxaloacetate. Rodriguez-Melendez et al. (144) reported less PCC and PC activities in the liver of biotin-deficient rats than in the liver of biotin-sufficient rats. Mock and Mock (106) also reported less PCC activity in the liver of biotin-deficient rats. Our main hypothesis was that biotin supplementation increases the activity and the expression of the genes for the biotin-dependent carboxylases PCC and PC in the liver of lactating dairy cows.

Increased activities of PC and PCC might result in increased production of oxaloacetate. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the decarboxylation of oxaloacetate to produce phosphoenolpyruvate (32,75), the precursor for glucose synthesis. Whereas phosphoenolpyruvate was synthesized in liver-extract incubations containing PC and PEPCK, little phosphoenolpyruvate was synthesized in incubations with PC only, therefore resulting in accumulation of oxaloacetate (75). Baird and Young (7) observed that the increase in the activity of PCC in the liver of dairy cows fed a diet containing 100% concentrate was accompanied by an increase in the activity of PEPCK. Because of its key role in gluconeogenesis, we expected an increased activity of PEPCK if biotin supplementation increases the production of oxaloacetate. Because the activity of PEPCK is correlated with PEPCK mRNA abundance in dairy cows (3), our
secondary hypothesis was that biotin supplementation would increase the gene expression of PEPCK in the liver of lactating dairy cows.

The objectives of this study were: 1) to determine the effect of biotin supplementation on the activity of PCC and PC in the liver of lactating high-producing dairy cows; and 2) to determine the effect of biotin supplementation on the expression of the genes for PCC, PC and PEPCK in the liver of lactating high-producing dairy cows.

MATERIALS AND METHODS

Materials and Reagents

NaH\(^{14}\text{C}\)CO\(_3\) (5.95 mmol/L, 8.4 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Propionyl-CoA (P5397), disodium ATP (A3377), sodium pyruvate (P2256), acetyl-CoA (A2056), NADH (N8129) and malate dehydrogenase (M7032) were purchased from Sigma (St. Louis, MO). Bradford Assay Kit (23236) was purchased from Pierce (Rockford, IL). Trizol\textsuperscript{®} reagent and reverse transcriptase enzyme (Super Script™ II) were purchased from Invitrogen Corporation (Carlsbad, CA). Ribonuclease inhibitor (RNasin\textsuperscript{®}, N2111) was purchased from Promega Corporation (Madison, WI). SYBR\textsuperscript{®} Green PCR master mix was purchased from Qiagen, Inc. (Valencia, CA). Random and specific primers were purchased from Operon Biotechnologies, Inc. (Huntsville, AL).
Animals and Diets

All procedures involving animals were approved by the Agricultural Animal Care and Use Committee of The Ohio State University. The experiment was designed as a cross-over design with two 22-d periods. Eight multiparous Holstein cows were fed a common diet (Table 4.1) for a 21-d preliminary period. At the end of the preliminary period, cows were at 162±35 d after parturition and producing 40±2 kg/d of milk. All cows were then randomly assigned to one of two sequences of treatments (Control→Biotin and Biotin→Control). Treatments consisted of an experimental diet (Table 4.1) with 0 (Control) or 0.96 mg (Biotin) of supplemental biotin per kg of dry matter (DM). The concentration of supplemental biotin was selected to supply 20 mg per day (90,98,198) based on an estimated DM intake of 22 kg/d. The diet was formulated to meet nutrient requirements (122) for a 650-kg lactating dairy cow producing 40 kg of milk per day. High proportions of grain in the substrate decreased microbial biotin synthesis in vitro (2); therefore, we formulated the basal diet to contain a high proportion of concentrate (60% DM basis). Supplemental biotin (Rovimix® Biotin, DSM Nutritional Products Inc., Parsippany, NJ) was supplied in the pelleted concentrate mix. Cows were housed in individual tie-stalls and fed once daily (0400 h). Diets were offered ad libitum (~5% feed refusals, as-fed basis) as mixed rations. Amount of feed offered and refused was measured daily. Diets were adjusted weekly for changes in ingredient DM concentration. Cows were milked twice daily (0200 and 1300 h) and milk weights were recorded electronically at each milking. The average of the daily milk yields from d 14 to d
20 was used as the value for milk yield. Cows were weighed on day 21 from each period at 0800 h.

Sample Collection and Analysis

Samples of feeds and feed refused were collected once weekly. An aliquot of feed and feed refusal samples were dried in a forced-air oven for 24 h at 100°C. Dry matter intake was measured as the difference between DM offered and DM refused. A second aliquot of feed was stored at -20°C until sample analysis. Forage samples were lyophilized and all feed samples were ground to pass through a 1-mm screen of a Wiley mill (Arthur A. Thomas, Philadelphia, PA). Ground samples were analyzed for DM (24 h at 100°C), neutral detergent fiber (Ankom\textsuperscript{200} Fiber Analyzer, Ankom Technology, Fairport, NY) with sodium sulfite and α-amylase (Ankom Technology), crude protein (Kjeldahl N × 6.25), ash (6) and starch (185).

Milk samples (a.m. and p.m. milkings) were collected on d 10 and 17 for determination of milk fat, protein and lactose concentrations with a B2000 Infrared Analyzer (Bentley Instruments, Chaska, MN) by DHI Cooperative, Inc. (Columbus, OH). Milk yields from d 10 and 17 were used to calculate yields of milk components.

An additional milk sample (p.m. milking), and blood and urine samples were collected on d 20. Concentrations of avidin-binding substances (ABS) in milk, plasma and urine, and ABS output in milk and urine were determined as described by Ferreira et al. (50). Concentration of ABS in liver tissue was also
determined. For this, crude liver homogenates (see *Activity of Biotin-dependent Carboxylases*) were diluted (1:20) before a single-step competitive enzyme binding assay (Ridascreen® Biotin kit, R-Biopharm Gmbh, Germany).

Samples of rumen fluid were collected on d 20 at 8:00 a.m. via stomach tube. Rumen fluid was processed and analyzed for volatile fatty acid concentration as described by Zimmerly and Weiss (198).

**Liver Biopsies**

On d 21 of each period liver tissue was collected by percutaneous liver biopsy. Liver biopsies were performed while cows were restrained in a squeeze chute. After clipping, locating liver parenchyma and large blood vessels by ultrasonography (5 MHz linear probe, Aloka 500V, Wallingford, CT) and appropriately scrubbing the area, 10 mL of 2% lidocaine hydrochloride was injected s.c. and i.m. at the 11th and 12th intercostal space. After making a 2-cm incision, a sterile biopsy instrument (5-mm internal diameter) was directed towards the opposite elbow and inserted through the muscle and peritoneal tissue into the liver. After retracting the biopsy instrument, the incision was closed with suture staples. Two aliquots of liver tissue were immediately rinsed with sterile 0.9% NaCl solution, placed in a sterile plastic vial, snap-frozen in liquid nitrogen and stored at –80°C.
Activity of Biotin-dependent Carboxylases

Liver tissue (0.86±0.22 g, wet weight) was homogenized as described by Zempleni et al. (18). Aliquots of the resulting supernatant (hereafter referred as liver homogenate) were snap-frozen in liquid nitrogen and stored at -80°C until enzyme activity and protein assays.

Activity of PCC was determined by measuring the fixation of $^{14}$CO$_2$ as described by Zempleni et al. (197) with modifications. Briefly, 5 µL of liver homogenate was vortex-mixed with 100 µL of pre-warmed (30°C) incubation buffer. The incubation buffer (169) contained 20 mmol/L NaH$^{[14]}$CO$_3$ (specific radioactivity 125 µCi/mmol), 100 mmol/L Tris (pH = 8.0), 0.75 mmol/L dithiothreitol, 6.0 mmol/L MgCl$_2$, 100 mmol/L KCl, 3.14 mmol/L disodium ATP, 1% Triton X-100 (v/v) and 1.0 mmol/L propionyl-CoA. The mixture was incubated in a water-bath at 30°C for 8 min. The reaction was terminated by adding 20 µL of 1 mol/L perchloric acid (vortex-mixed). A 100-µL aliquot was transferred into 5-mL scintillation vials, dried in a dry-bath at 55°C to remove unfixed CO$_2$ for approximately two hours (i.e., until a purplish residue was observed) and mixed with 4 mL of scintillation liquid (Ecolite +, MP Biomedicals, Irvine, CA). Fixed $^{14}$CO$_2$ was measured using a liquid scintillation analyzer (BS 6500, Beckman Coulter, Inc., Fullerton, CA). Final values were obtained after a 96-h equilibration of the dried residue with the scintillation liquid.

Activity of PC was determined by measuring the fixation of $^{14}$CO$_2$ as described by Zempleni et al. (197) with modifications. Briefly, 5 µL of liver homogenate was vortex-mixed with 100 µL of pre-warmed (30°C) incubation
buffer. The incubation buffer (181) contained 15 mmol/L NaH[\(^{14}\)C]O\(_3\) (specific radioactivity 680 μCi/mmol), 100 mmol/L Tris (pH = 8.0), 5 mmol/L MgSO\(_4\), 0.1 mmol/L acetyl-CoA, 0.1 mmol/L NADH, malate dehydrogenase (5-units per mL), 1 mmol/L disodium ATP and 5 mmol/L sodium pyruvate. The mixture was incubated in a water-bath at 30°C for 5 min. The reaction was terminated and fixed \(^{14}\)CO\(_2\) was measured as described above.

Blank reactions were performed in all assays by omitting liver homogenate in the incubations. All samples were assayed in triplicate. The CV among triplicates were 9.6 and 10.0% for PCC and PC, respectively. All specific activities are expressed in nmol of total CO\(_2\) fixed per mg protein in one min at 30°C.

Protein concentration in liver homogenates was determined in triplicates according to Bradford’s method using BSA as a protein standard. The CV among triplicates was 5.3%.

**RNA Extraction**

Total RNA was isolated from liver tissue (0.35±0.11 g, wet weight) using 4-mL of Trizol reagent following manufacturer’s instructions. Precipitated RNA was redissolved in 400 μL RNase-free water. Protein contamination of the isolated RNA was determined by the ratio of optical absorbance at 260-280 nm, which averaged 1.66±0.05.
Quantification of mRNA

Reverse-transcription (RT) reaction was performed using Super Script II reverse transcriptase following manufacturer’s instructions, but using RNasin as the ribonuclease inhibitor. To control for DNA contamination in the extracted RNA, RT reactions were also performed without the addition of reverse transcriptase enzyme. No products were obtained.

Primers for PC, PCC and cytosolic PEPCK were designed after obtaining bovine-specific mRNA sequences from the National Center of Biotechnology Information (Table 4.2). Primers were diluted to a working concentration of 15 µmol/L. Quantitative real-time PCR was performed in 96-well plates by incubating 2 µL of RT product with 0.5 µL of forward primer, 0.5 µL of reverse primer, 10 µL of SYBR Green PCR master mix and 7 µL of RNAse-free water. Quantitative real-time PCR was performed in a continuous fluorescence detector (DNA Engine Opticon 2 System, Bio-Rad Laboratories, Inc., Waltham, MA) using the following temperatures and times per cycle (40 cycles): denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 60 s. The first cycle was preceded by denaturation at 95°C for 15 m. Non-specific amplification was not evidenced by melting curve analysis and agarose-gel electrophoresis. Primer pairs were also validated after sequencing the amplified product.

For each gene, all samples (n = 16) were amplified in triplicate and in one plate (i.e., within run replication). Expression of the genes for PC, PCC and PEPCK were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (Table 4.2).
**Statistical Analysis**

All variables were analyzed using the MIXED procedure of SAS (SAS Institute, Inc., Cary, NC). The model included the effects of cow (random, df = 7), diet (fixed, df = 1), period (fixed, df = 1), the diet by period interaction (fixed, df = 1) and the residual error (random, df = 5). Liver biopsy of Period 1 may have affected certain variables (e.g., DM intake and milk yield) during Period 2, therefore, we considered period as a fixed effect.

**RESULTS**

Biotin supplementation did not affect DM intake, milk yield, or milk composition (Table 4.3). Daily DM intake decreased 2.2 kg from period 1 to period 2. Accordingly, daily milk yield decreased 4.2 kg from period 1 to period 2. Consistent with previous studies (50,198), biotin supplementation did not affect molar proportions of volatile fatty acids in the rumen (59.7, 22.2, 13.8, 0.8, 2.4 and 1.1 mol/100 mol VFA for acetate, propionate, butyrate, isobutyrate, valerate and isovalerate, respectively).

Biotin supplementation increased the concentration of ABS in plasma, milk and urine, and the daily output of ABS in milk and urine, but did not affect the concentration of ABS in liver tissue (Table 4.4).

Biotin supplementation tended to increase (P < 0.12) the activity of PC (Figure 4.1), but did not affect (P > 0.20) the activity of PCC in liver tissue.
Biotin supplementation did not affect \( P > 0.20 \) the expression of the genes for PC, PCC and PEPCK (Figure 4.3).

**DISCUSSION**

In this study we postulated that increased gluconeogenesis in the liver is a potential mechanism by which biotin supplementation increases milk yield by dairy cows. More specifically, we hypothesized that biotin supplementation increases the activities of PC and PCC, and the gene expression of PC, PCC and PEPCK. Contrary to our expectations, biotin supplementation did not affect the activity of PCC and the gene expression of the genes for PC, PCC and PEPCK, but tended to increase the activity of PC. Lower PC (144) and PCC (106,144) activities were observed in liver from rats fed a biotin-deficient diet than in liver from rats fed a biotin sufficient diet.

The activities of PC and PCC were similar to those previously reported for dairy cows (120), but substantially greater than those reported in the liver of rats (197). The different activities between dairy cows and rats may be related to differences in the role of gluconeogenesis in the metabolism these species. Contrary to non-ruminants, net flux of glucose across portal-drained viscera in lactating dairy cows is typically negligible or negative (141,142), suggesting that there is little or no net absorption of glucose from the gastrointestinal tract. However, the net flux of glucose from total splanchnic tissues in lactating dairy cows is typically positive (141,142). This is due to the capacity of the liver to
synthesize glucose from propionate, lactate, glycerol and some glucogenic amino acids. While PC uses lactate, glycerol and alanine, PCC uses propionate as the carbon source for gluconeogenesis (123).

The reasons why biotin increased \( P < 0.12 \) the activity of PC and not the activity of PCC are not clear. Even though lactate, glycerol and alanine are important precursors for glucose, propionate alone accounts for 55 to 90% of the glucose carbon in lactating dairy cows (141,142). Because glucose supply to the mammary gland relies greatly on gluconeogenesis from propionate, PCC may have a higher priority for biotin than PC.

Because biotin supplementation did not affect the expression of the gene for PC, the increased activity of PC may be attributed to an increased activation of the apocarboxylase into holocarboxylase by action of the enzyme holocarboxylase synthetase (HCS). Rodriguez-Melendez et al. (144) observed a lower activity of PC in the liver of biotin-deficient rats, while the mRNA abundance for PC in the liver was similar between biotin-deficient and biotin sufficient-rats. Rodriguez-Melendez et al. (144) and Solorzano-Vargas et al. (164) reported that biotin deficiency decreased the mRNA abundance of HCS in rats and humans. In this study we attempted to measure the abundance of HCS mRNA as a marker of HCS activity, although no message amplification was obtained using three different primer pairs.

Contrary to our second hypothesis, biotin supplementation did not affect the expression of PEPCK in the liver of lactating dairy cows. We based our hypothesis on the assumption that an increased activity of PEPCK would be
required for the conversion of the additional oxaloacetate (i.e., the presumed result of increased activities of PCC and PC). Accordingly, Baird and Young (7) indicated that the activity of PEPCK in the liver of dairy cows responded to dietary changes (50 vs. 100% of concentrate in the diet), likely in response to an increased load of propionate to the liver. Biotin supplementation may not have increased the substrate load enough to increase the activity of PEPCK. Also, the mRNA abundance of PEPCK may not be an adequate indicator of PEPCK activity. Contrary to previous observations (3), no correlation between the activity and the mRNA abundance of PEPCK in liver from calves has been reported recently (63).

Even though it increased the concentration of ABS in plasma, milk and urine, biotin supplementation did not affect the concentration of ABS in the liver (Table 4.4). Lewis et al. (87), also reported that rats fed a control diet (non-supplemented) or a biotin-supplemented diet had similar concentrations of free-biotin in the liver. The reasons for the similar concentration of ABS in liver tissue are not clear. Biotin is transported into hepatocytes by a sodium-dependent transporter that has a $K_m$ in the micromolar range (154). Based on this transport system, biotin uptake by the liver would likely be related to biotin concentration in plasma.

Residual or carry-over effects may affect interpretations when a sequence of treatments is applied to the same subject, such as in the case of Latin square and cross-over designs. Whether carry-over of biotin existed in this experiment is not certain, but seems unlikely. Frigg et al. (53) measured the clearance of an
oral dose of biotin (40 mg) in dairy heifers, and observed that biotin concentration in plasma returned to baseline concentrations 72 h after dosing biotin. Accordingly, during period 2, the concentrations of ABS in plasma, milk and urine of cows consuming the Control diet were similar to those of cows consuming the Control diet during period 1 (Table 4.4). Weinberg and Utter (184) reported that the half-life for PC in rat liver was 4.6 days. Assuming that this value is similar for carboxylases in liver of dairy cows, residual effect on the enzyme activity would also be unlikely.

Biotin supplementation did not affect any of the production measures in this study (Table 4.3). These observations were not expected and are inconsistent with previous observations (50). However, DM intake (1.7 and 2.7 kg/d) and milk yield (4.3 and 4.1 kg/d) decreased substantially from period 1 to period 2 (Table 4.3). A ≥4-kg/d decrease in milk yield is much greater than typical decreases for 21-d periods. The likeliest reason for this decrease is that liver biopsies from period 1 influenced DM intake and lactation performance during period 2. Rectal temperatures were monitored during the five days following biopsies, and no readings greater than 39.4°C were observed. Despite this, DM intake and milk yield were clearly reduced after liver biopsies from period 1 (Figure 4.4). This observation and the limited number of cows limit our interpretations of production data.

In conclusion, biotin supplementation did not affect the activity of PCC and the expression of the genes for PCC, PC and PEPCK in the liver of high-producing dairy cows, but tended to increase the activity of PC. The differential
response to biotin supplementation suggests that PCC may have a higher priority for biotin than PC. Based on our data, the increase in milk production by lactating dairy cows supplemented with biotin may be related to an increase in biotin-dependent carboxylases. Whether biotin supplementation increases glucose production in the liver still needs to be evaluated.

ACKNOWLEDGMENTS

Appreciation is extended to Dr. Juliette Hanson for her assistance with the liver biopsies and to the crew of Krauss Dairy Center at the OARDC for the day-to-day cow management and their assistance with liver biopsies. Gratitude is also extended to Dr. Janos Zempleni for his guidance for enzyme activity assays and to Mrs. Jodi Winkler for her assistance with quantitative real-time PCR.
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Preliminary</th>
<th>Experimental&lt;sup&gt;1,2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
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<td>Corn silage</td>
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<td>12.1</td>
</tr>
<tr>
<td>Alfalfa silage</td>
<td>31.1</td>
<td>30.4</td>
</tr>
<tr>
<td>Corn grain (dry and ground)</td>
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<td>20.7</td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>8.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>13.7</td>
<td>16.3</td>
</tr>
<tr>
<td>Soybean meal (44% crude protein)</td>
<td>6.4</td>
<td>7.7</td>
</tr>
<tr>
<td>Fat (animal and vegetable blend)</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Urea</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Minerals and vitamins</td>
<td>1.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**Chemical analysis**

<table>
<thead>
<tr>
<th></th>
<th>Preliminary</th>
<th>Experimental&lt;sup&gt;1,2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>20.6</td>
<td>20.6</td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
<td>33.4</td>
<td>33.5</td>
</tr>
<tr>
<td>Starch</td>
<td>19.9</td>
<td>19.4</td>
</tr>
<tr>
<td>Ash</td>
<td>6.6</td>
<td>6.6</td>
</tr>
</tbody>
</table>

<sup>1</sup> Experimental diet with supplemental biotin contained 0.0048% of biotin premix (Rovimix<sup>®</sup> H-2, 20 mg/g), which replaced a similar amount of corn gluten meal.

<sup>2</sup> The diet contained 0.38% dicalcium phosphate, 0.58% limestone, 0.01% magnesium oxide, 0.20% trace mineral salt, 0.15% selenium (200 mg/kg), 0.0023% zinc sulfate, 0.0012% copper sulfate, 3,000 IU/kg vitamin A, 870 IU/kg vitamin D, and 21 IU/kg vitamin E.

**Table 4.1.** Ingredients and nutrient composition (%) DM basis of the basal diet.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>NM_177946</td>
<td>F 5'-CATGCGCTTCCTGTACGAGT-3'</td>
<td>1846</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5'-CGCAGAACTTGAAGACCACA-3'</td>
<td>1991</td>
</tr>
<tr>
<td>PCC</td>
<td>CB223151</td>
<td>F 5'-GAGGACACAAGCAGCATTCT-3'</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5'-GCAGGTCTCCTTCTCAAACT-3'</td>
<td>454</td>
</tr>
<tr>
<td>PEPCK</td>
<td>NM_174737</td>
<td>F 5'-TCCTTGGGAAGAAGTGCT-3'</td>
<td>388</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5'-CGGGAGAGTAGGGTTTCAT-3'</td>
<td>572</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_001034034</td>
<td>F 5'-AAGATTGTAGGAATGCT-3'</td>
<td>499</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5'-ACAGACACGTTGGAG-3'</td>
<td>791</td>
</tr>
</tbody>
</table>

1 PC = pyruvate carboxylase; PCC = propionyl-CoA carboxylase; PEPCK = phosphoenolpyruvate carboxykinase; GAPDH = glyceraldehydes-3-phosphate dehydrogenase.

**Table 4.2.** Primer design for quantitative RT-PCR.
Table 4.3. Body weight, dry matter (DM) intake and production of cows consuming a diet containing 0 (Control) or 0.96 mg/kg DM (Biotin) of supplemental biotin.
### Table 4.4

Concentration of avidin-binding substances (ABS) in plasma, milk, urine and liver tissue of cows consuming a diet containing 0 (Control) or 0.96 mg/kg DM (Biotin) of supplemental biotin.

<table>
<thead>
<tr>
<th></th>
<th>Period 1</th>
<th>Period 2</th>
<th>SEM</th>
<th>D</th>
<th>P</th>
<th>P×B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ABS, nmol/L</td>
<td>Control</td>
<td>Biotin</td>
<td>Control</td>
<td>Biotin</td>
<td></td>
<td>SEM</td>
</tr>
<tr>
<td></td>
<td>3.74</td>
<td>4.65</td>
<td>3.24</td>
<td>5.27</td>
<td>0.50</td>
<td>0.01</td>
</tr>
<tr>
<td>Milk ABS, nmol/L</td>
<td>117</td>
<td>331</td>
<td>102</td>
<td>327</td>
<td>34</td>
<td>0.01</td>
</tr>
<tr>
<td>Milk ABS output, μmol/d</td>
<td>4.2</td>
<td>14.5</td>
<td>3.8</td>
<td>10.9</td>
<td>1.44</td>
<td>0.01</td>
</tr>
<tr>
<td>Urine ABS, μmol/mol creatinine</td>
<td>73</td>
<td>218</td>
<td>96</td>
<td>141</td>
<td>18.8</td>
<td>0.01</td>
</tr>
<tr>
<td>Urine ABS output, μmol/d</td>
<td>6.6</td>
<td>21.2</td>
<td>9.3</td>
<td>13.2</td>
<td>1.83</td>
<td>0.01</td>
</tr>
<tr>
<td>Liver ABS, pmol/mg protein</td>
<td>0.97</td>
<td>0.96</td>
<td>1.12</td>
<td>1.17</td>
<td>0.15</td>
<td>0.92</td>
</tr>
</tbody>
</table>

1 D = effect of diet; P = effect of period; P × B = interaction of diet by period.

2 Estimated assuming a creatinine excretion of 29 mg per kg of body weight (177).
Figure 4.1. Enzyme activity of pyruvate carboxylase in the liver of lactating dairy cows. Cows were fed a diet containing 0 (Control) or 0.96 mg/kg DM (Biotin) of supplemental biotin. Values are means ± SEM. Diet effect ($P < 0.12$); interaction of diet by period ($P < 0.18$).
Figure 4.2. Enzyme activity of propionyl-CoA carboxylase in the liver of lactating dairy cows. Cows were fed a diet containing 0 (Control) or 0.96 mg/kg DM (Biotin) of supplemental biotin. Values are means ± SEM.
Figure 4.3. Gene expression of pyruvate carboxylase (PC), propionyl-CoA carboxylases (PCC) and phosphoenolpyruvate carboxykinase (PEPCK) in the liver of lactating dairy cows. Cows were fed a diet containing 0 (Control) or 0.96 mg/kg DM (Biotin) of supplemental biotin. Abundance of mRNA was normalized using glyceraldehyde-3-phosphate dehydrogenase. Values are means ± SEM.
Figure 4.4. Mean dry matter intake (bold line) and milk yield (thin line) throughout the experiment. Arrows indicate days at which liver biopsies were performed. Values are the average of all cows (n = 8).
CHAPTER 5

CONCLUSIONS

This dissertation contributed to our understanding of when and how biotin supplementation increases milk production by lactating dairy cows in different ways. From the first experiment we concluded that biotin supplementation increases milk yield in high-producing, but not in low-producing cows. This is very important information for nutritionists and dairy farmers. Based on the current low cost of biotin and the beneficial effect on foot health, Shearer (162) suggested that biotin supplementation in lactating dairy cows is worthy of consideration. Even though I agree with his suggestion, the use of biotin would be more cost-effective by supplementing it to high-producing cows than to low-producing cows.

A response in milk yield to biotin supplementation suggests that biotin nutritional status is not always optimal for lactation performance. In the first experiment we also evaluated and concluded that the concentration of avidin-binding substances in plasma and milk (two typical measures of biotin status in animal trials) do not reflect differences in biotin nutritional status. The first experiment also showed that, different to non-ruminants, the urinary excretion of 3-hydroxyisovaleric acid is not a sensitive determinant of biotin status for
lactating dairy cows. Future studies should focus on finding other measures for
dairy cows. The relative concentrations of biotin and biotin metabolites in blood,
milk or urine are potential determinants that need to be evaluated. However,
review of the literature and data from our laboratory (unreported experiments)
show that quantifying biotin and biotin metabolites is not easy. Therefore, efforts
should also focus on other alternatives. The activity of propionyl-CoA
carboxylase in lymphocytes is a sensitive determinant of biotin status in non-
ruminants (106). My opinion is that measuring the enzyme activity of biotin-
dependant carboxylases in lymphocytes may be a suitable determinant of biotin
status in dairy cows. One characteristic of lymphocytes is that biotin uptake is not
through a SMVT system, but through a transporter system that is saturable at
physiological concentrations (196). Maybe the activity of propionyl-CoA
carboxylase in lymphocytes is related to biotin uptake by this system. Whether
this would apply for dairy cows is not known. Although the use of radioisotopes is
discouraged, measuring the enzyme activity of biotin-dependent carboxylases is
simple. It would be interesting to see whether this measure is a sensitive
determinant of biotin status for dairy cows as it is for non-ruminants (106).

Also, before this research no information existed regarding the mechanism
by which biotin supplementation increases milk yield by dairy cows. In the
second study we observed that biotin supplementation can increase the activity
of biotin-dependent gluconeogenic carboxylases in the liver of lactating dairy
cows. This information is the first direct evidence that biotin supplementation may
increase gluconeogenesis. Future studies should evaluate whether biotin supplementation in fact has an effect on glucose production.

Finally, this dissertation also raises some concerns about our current knowledge of the metabolism of biotin in lactating dairy cows. From the review of literature I conclude that there are many areas of the metabolism of biotin in ruminants in which our knowledge is quite limiting. Even though there is evidence that it is synthesized in the rumen, we know little about how much of the total biotin supplied to dairy cows is originated from microbial synthesis. Future research should focus on identifying microbial species from the rumen that are capable of synthesizing biotin and determining which factors (e.g., dietary factors) affect the distribution of different microbial populations. Also, little is known about the mechanism of absorption and degradation of biotin in the gastrointestinal tract. Knowledge in these areas will substantiate our understanding of the metabolism of biotin in lactating dairy cows, and therefore should also be explored in the future.
REFERENCES

1. The Ohio State University Extension. Questions pertaining to large dairy enterprises in Ohio: General information about the dairy industry. Extension FactSheet AS-12-03.


140. Reynolds CK, Huntington GB, Tyrrell HF, Reynolds PJ. **1988**. Net metabolism of volatile fatty acids, D-beta-hydroxybutyrate, nonesterifield


Figure A.1. Milk yield of cows before (Days -7 to -1), during (Days 1 to 14) and after (Days 15 to 28) the experiment (data from Experiment 1).