Indicators of marginal biotin deficiency and repletion in humans: validation of 3-hydroxyisovaleric acid excretion and a leucine challenge[1–3]

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ABSTRACT

Background: The results of clinical studies have provided evidence that marginal biotin deficiency is more common than was previously thought. A previous study of 10 subjects showed that the urinary excretion of biotin and 3-hydroxyisovaleric acid (3HIA) are early and sensitive indicators of marginal biotin deficiency.

Objective: Marginal biotin deficiency was experimentally induced and corrected to assess the utility of 3 indicators of biotin status: urinary excretion of biotin and 3HIA and the increase in 3HIA excretion after leucine loading.

Design: Eleven healthy adults consumed an egg white diet for 28 d. Blood and 24-h urine samples were collected before the start of the diet and twice weekly thereafter. In 5 subjects, an oral leucine challenge was performed weekly for 4 wk. After depletion, biotin status was restored with a general diet with or without a supplement containing 80 μg biotin. Urinary excretion of biotin, bisnorbiotin, and biotin sulfoxides was determined by avidin-binding assay after HPLC. Excretion of 3HIA, an indicator of reduced activity of the biotin-dependent enzyme methylcrotonyl-CoA carboxylase (EC 6.4.1.4), was measured by gas chromatography–mass spectrometry.

Results: 3HIA excretion increased significantly with time on the egg white diet ($P < 0.0001$), as did 3HIA excretion in response to the leucine challenge ($P < 0.002$); the excretion of both biotin and bisnorbiotin decreased significantly with time ($P < 0.0001$). In most subjects, biotin status returned to normal after 1 wk of a general diet.

Conclusions: Excretion of 3HIA and of biotin are early and sensitive indicators of biotin deficiency. 3HIA excretion after a leucine challenge is at least as sensitive. *Am J Clin Nutr* 2002;76:1061–8.

KEY WORDS Biotin, leucine, metabolism, 3-hydroxyisovaleric acid, bisnorbiotin, egg whites

INTRODUCTION

The results of clinical studies conducted by our group and others have provided evidence that marginal biotin deficiency in such disparate clinical circumstances as pregnancy (1, 2), protein-energy malnutrition (3), and long-term therapy with certain anticonvulsants (4–8) is not rare. As noted in an editorial in this journal, there is a need to develop valid indicators of marginal biotin deficiency (9).

In 1997 we reported the results of the first experimental study to successfully induce biotin deficiency in human subjects since the 1940s. In that study, we evaluated 3 indexes of biotin status that depended on the previous development of 2 analytic methods: *1*) a sensitive, chemically specific assay for biotin that combines HPLC with an avidin-binding assay and *2*) a gas chromatography–mass spectrometry method for measuring 3-hydroxyisovaleric acid (3HIA) in urine (10).

Biotin is a covalently bound prosthetic group for 4 mammalian carboxylases; one of these, methylcrotonyl-CoA carboxylase (EC 6.4.1.4), catalyzes an essential step in the intermediary metabolism of the branched-chain amino acid leucine. Decreased activity of methylcrotonyl-CoA carboxylase shunts the substrate 3-methylcrotonyl CoA to an alternate metabolic pathway, producing 3HIA, which is then excreted in urine.

In that first study, we observed that decreased urinary excretion of biotin was an early and sensitive indicator of biotin deficiency; biotin deficiency was identified before the onset of symptoms and was detected in almost all subjects. However, a decrease in the plasma concentration of biotin was detected in less than one-half of the subjects. Increased urinary excretion of 3HIA was also an early and sensitive indicator of biotin deficiency.

Although that study was a reasonable first effort, the conclusions were limited in the following ways. *1*) Homogeneity of biotin status was not established with a biotin loading-washout phase. *2*) Only 10 subjects were studied (7 men, 3 women). *3*) Recovery from deficiency was not studied in the repletion phase. We addressed these limitations in the study reported here and expanded the scope of the study to include evaluation of an oral leucine challenge as an index of biotin status.

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TABLE 1
Calculated nutrient composition of the 2 diets[1]

Nutrient	Menu 1	Menu 2
Protein (g)	129	186
Leucine (g)	9	13
Fat (g)	68	99
Carbohydrate (g)	273	395
Dietary fiber (g)	22	31
Cholesterol (mg)	74	107
Biotin (μg)	23	13.4

[1] Amount per 10 MJ.

SUBJECTS AND METHODS

Study subjects

All human studies were approved by the University of Arkansas for Medical Sciences' Human Research Advisory Committee; informed consent was obtained at enrollment. Initially, 15 healthy adult volunteers (10 women) resided in a general clinical research center (GCRC) for 28 d. The subjects were allowed to go to work on weekdays and took the noon meal and urine collection vessels with them; otherwise, the subjects resided fulltime at the GCRC.

Individuals who were supplementing their diets deliberately or inadvertently with dietary biotin were excluded from enrolling. Examples of supplements that substantially increase biotin intake include many breakfast cereals, weight-gain supplements, weight-loss supplements, and multivitamin supplements. These foods contain amounts of biotin that are substantial compared with the estimated daily dietary intake of 35–70 μg/d (143–287 nmol/d) (11–15). Because biotin is often present in foods covalently bound to protein and thus is difficult to measure, and because most assays do not discriminate between biotin and its various metabolites, the estimates of biotin content are not precise. Thus, potential volunteers taking any type of supplement were excluded unless negligible biotin content was confirmed by assay.

Eleven subjects (8 women) completed the study. The 4 subjects who did not complete the study withdrew during the biotin depletion phase of the study due to difficulties in complying with the diet and housing protocols.

Study design

Loading and washout

On study day −14, the subjects began the loading phase by receiving a daily supplement containing 300 μg biotin (1.2 μmol). This supplement is 10 times the recommended adequate intake of 30 μg (16). In addition, a daily vitamin supplement not containing biotin was given. This supplement contained vitamin A (5000 IU), vitamin C (60 mg, or 340 μmol), vitamin D (400 IU), vitamin E (15 IU), thiamine (1.5 mg, or 5 μmol), riboflavin (1.7 mg, or 5 μmol), niacin (20 mg, or 163 μmol), vitamin B-6 (2 mg, or 10 μmol), folate (400 μg, or 1 μmol), vitamin B-12 (6 μg, or 4 nmol), and iron (18 mg, or 322 μmol).

On study day −7, the biotin supplement was discontinued to begin the washout period. The multivitamin supplement without biotin was continued through study day 28. The amount of biotin in the multivitamin supplement without biotin was measured by direct assay with the avidin-binding assay (17). The mean biotin content was 0.01 μg (48 pmol); the range was between 0.004 and 0.03 μg (18–120 pmol; $n = 4$). This amount is < 0.09% of the estimated daily dietary intake of 35–70 μg/d (143–287 nmol/d) (11, 12) and 0.3% of the study dietary intake of 10.2 μg (42 nmol) for women and 13.8 μg (56 nmol) for men.

Biotin depletion

On study day −1, the subjects were admitted to the GCRC. They provided a 24-h urine collection, which served a test run for proper collection technique. A urine chorionic gonadotropin pregnancy test was performed on the samples from the women and all test results were negative. On study day −1, the subjects received a self-selected, general diet. At 0730 of study day 0, the subjects completed a second 24-h urine collection.

Feeding of the research diet (referred to hereafter as the egg white diet) commenced on study day 0. The egg white was provided as dried albumen (Wakefield Brand; M.G. Waldbaum, Wakefield, NE) in a blended beverage containing 16 g dried egg white/MJ dietary energy; the egg white accounted for 22–32% of the diet's dry weight, depending on consumption of the alternate diets described below. This egg white content successfully produced biotin deficiency in the previous study (10). The egg white content was chosen to contain sufficient avidin to bind ≈78 times the dietary biotin intake, which was calculated from estimates of biotin in food sources (11, 18, 19) and was measured directly in our laboratory as described below. The calculation used a published value for the avidin content of egg white (20, 21), and the estimate of binding capacity used an avidin-to-biotin binding ratio of 1:4.

The egg white beverage was consumed at breakfast (25%), lunch (33%), and dinner (42%). Energy expenditure for each volunteer was calculated by using the Harris Benedict equation (22) to provide a euenergetic menu. The typical daily energy intake was 9700 kJ for women and 14 000 kJ for men: 23% from protein, 50% from carbohydrate, and 27% from fat; nutrient content was estimated by using NUTRITIONIST 5, version 1.6 (First Data Bank, San Bruno, CA). The typical American diet provides ≈12–15% protein, 45–50% carbohydrate, and 35–50% fat. The egg white beverage was provided in proportion to the energy content of each meal; because high-biotin foods were avoided in the protocol meals, energy content roughly paralleled biotin content. Nutrient concentrations were as shown in **Table 1** as menu 1 and menu 2.

Subjects received a 2-d rotating menu cycle. To increase variety, the foods in each menu were rearranged to produce 2 additional menu cycles. For each subject, food amounts were factored proportionately to provide equal daily energy intake. Foods were weighed to the nearest gram. Subjects were allowed up to 12 oz (360 mL) coffee, tea, or diet soda throughout the day. Dietary compliance was judged by observing meals eaten, by inquiring daily about other foods consumed, by daily morning weigh-ins, and by subjects completing daily journals kept in the GCRC. Subjects were required to consume the entire egg beverage with each meal. Although subjects were strongly encouraged to consume each meal entirely, they were allowed to refuse portions of foods. Uneaten food was recorded; data for actual food consumption were used to calculate average nutrient intake.

Repletion phase

On study day 28, the egg white diet was discontinued, and all subjects consumed a self-selected, mixed general diet from study day 28 to 49. For the first week of repletion (study day 28 to 35), 5 subjects received a daily multivitamin and multimineral supplement with biotin. For these subjects, the intent was to provide a supplement that contained an amount of biotin (30 μg) equal to

the adequate intake. The labeled content was 30 μg biotin (123 nmol), and our previous measurements have indicated that labeled biotin content in commercial vitamins is typically accurate within 10% (17). However, analysis of the multivitamin after the subjects began consuming the supplement showed that the biotin content was greater than the labeled amount: 80 ± 13 μg (328 ± 53 nmol; ± SD; $n = 5$). The supplement contained the same vitamin content as the biotin-free supplement with the following exceptions: vitamin K (25 μg, or 0.1 μmol), vitamin B-6 (2.9 mg, or 14 μmol), and pantothenic acid (10 mg, or 46 μmol).

Six subjects continued to receive the daily multivitamin supplement without biotin through study day 35. Thereafter, all subjects received the 80-μg biotin supplement.

Leucine dose selection

In support of the main study described here, we empirically established a suitable dose of leucine for the leucine challenge tests by conducting a small leucine-dose-ranging study. In this study, the biotin status of 3 healthy adults was altered as follows: lowered biotin status (induced by avidin feeding), normal biotin status (mixed general diet), and augmented biotin status (produced by supplementation with 300 μg biotin/d). Individuals were studied at 2 leucine doses. After providing a baseline void, subjects drank the leucine dose suspended in water; subsequent voids were collected at 2-h intervals for 6 h.

Leucine challenge

In the depletion phase of the main study, 5 subjects underwent a leucine challenge on study days 1, 7, 14, and 21. These 5 subjects consumed 70 mg leucine/kg (534 μmol/kg) dissolved in orange juice after collecting the last void of the previous 24-h collection. All voids were collected for 5 h after ingestion and pooled. No other food was consumed during this 5-h period. The full egg white diet for the day, including the egg white beverage, was consumed later in the day.

Sample collection and handling for biotin and 3-hydroxyisovaleric acid excretion

In addition to the collection on study day 0 as noted above, 24-h urine samples were collected on study days 3, 7, 10, 14, 17, 21, 24, 28, 35, and 49. During 24-h urine collections, individual voids were pooled and refrigerated immediately; no preservative was added. Before storage, urine samples were warmed to 50 °C for 30 min, centrifuged (10 min at 1200 × g and room temperature) to remove any particulates, and portioned for storage at −20 °C until analyzed.

The completeness of each urine collection was evaluated by determining the total creatinine excretion per 24 h. Creatinine excretion was not less than the lower limit of the normal range for any collection. The normal range for men and women established in our laboratory was similar to published normal ranges.

Analytic methods

The presence of substantial amounts of biotin metabolites in human urine and plasma in subjects who are biotin deficient, biotin adequate, and biotin supplemented (10, 23–27) requires the use of a chemically specific assay. To specifically measure concentrations of biotin and biotin metabolites, we used a 2-step approach. First, biotin and the biotin metabolites were separated by C_{18} reversed-phase chromatography (28); next, each compound was quantitated against the authentic biotin analogue. Authentic standards were required because the various biotin analogues differ in avidin-binding affinity (21). For this study, HPLC retention times were determined by using radiolabeled biotin, bisnorbiotin, and biotin sulfoxides; the appropriate HPLC fractions were collected and analyzed against authentic standards as described previously (24, 29). The term *sulfoxides* is used because the *d* and *l* isomers are not resolved by this HPLC method.

The urinary excretion of 3HIA was determined by gas chromatography–mass spectrometry (10). This method uses authentic unlabeled and deuterated 3HIA as the external and internal standards, respectively. Creatinine was measured in our laboratory by the picric acid method (30, 31) with a Beckman Creatinine Analyzer 2 (Beckman Instruments, Inc, Brea, CA).

Statistics

Normal ranges

Over the past decade, for each study of biotin status within a subject population, our laboratory has recruited individuals to serve as control subjects. As our understanding of biotin nutriture has grown, we have reanalyzed our stored samples with the use of improved techniques and have identified factors that disqualify some individuals from participating as control subjects, eg, the use of birth control medication. Taken together, these results provide a reasonable normal range with which to compare the results from the current study. Despite the exclusion of individuals who acknowledged biotin supplementation, our measurements of the biotin excretion of free-living subjects showed a bimodal distribution; we speculate that the small subgroup that excreted larger amounts of biotin inadvertently or deliberately supplemented their biotin intake. Because the distribution was not normal, the normal range for biotin excretion (19–62 nmol/d) was chosen as the 10th percentile to the 90th percentile of 19 subjects (11 women). A similar approach was used to establish the normal ranges for bisnorbiotin (12–54 nmol/d) and biotin sulfoxides (6–15 nmol/d) by using values from the same subjects. The normal range for the excretion of 3HIA (39–150 μmol/d) was determined from 17 subjects (9 women); the distribution was monomodal and roughly normal.

Comparison of subjects at study day 0 with control subjects (ie, not subjected to prior biotin load and washout)

For nutritional status indicators with normal distributions, the significance of differences between subjects was tested by using Student's unpaired, two-tailed *t* test with significance set at $P < 0.05$. For indicators with nonnormal distributions, the significance of differences was tested by using the Mann Whitney *U* test with significance set at $P < 0.05$.

Studies of 3-hydroxyisovaleric acid excretion for baseline and leucine challenge

The significance of differences in baseline 3HIA excretion among the individuals with different biotin status was tested by one-way analysis of variance (ANOVA). The significance of differences in challenge 3HIA excretion among the individuals with different biotin status was also tested by one-way ANOVA. If overall differences were significant at $P < 0.05$, Fisher's post hoc test with Bonferroni correction was used to define pairwise differences.

Testing of trends with duration of egg white feeding and repletion

The significance of trends with duration of egg white feeding was tested by one-way ANOVA with repeated measures.

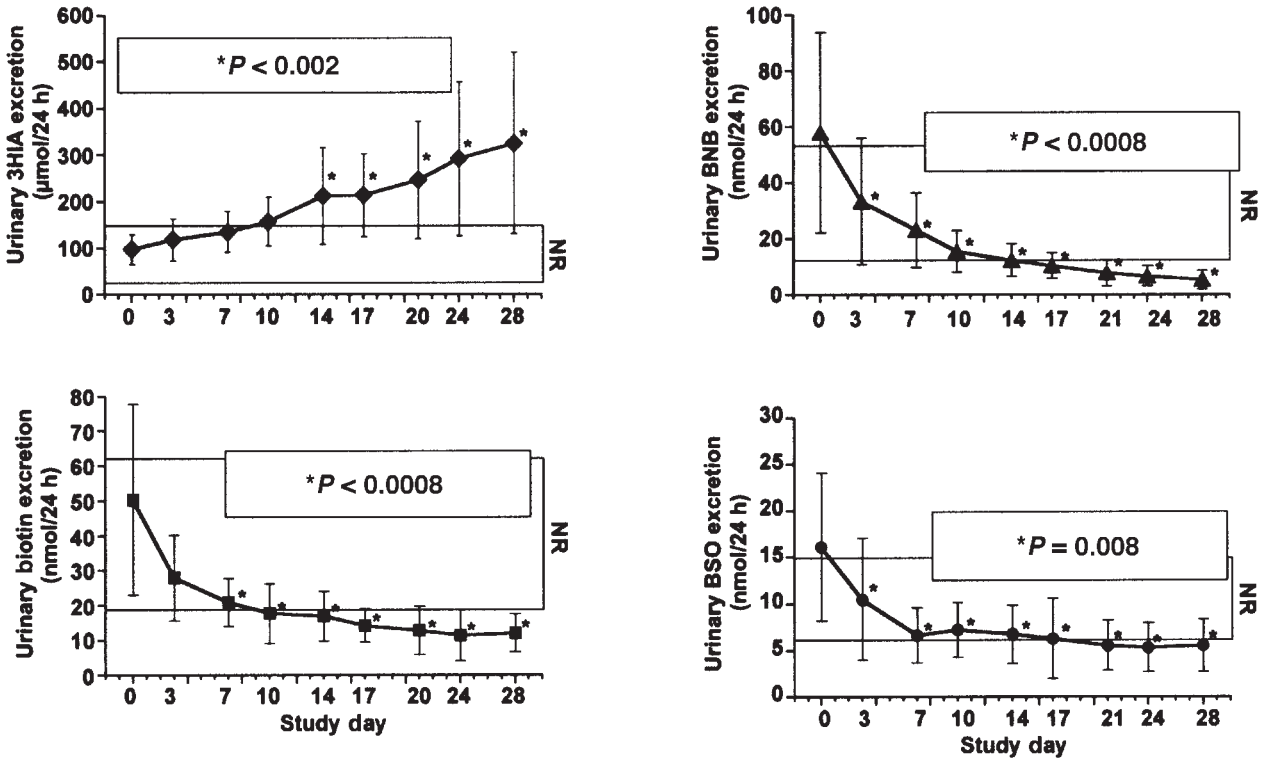

FIGURE 1. Depletion phase. Mean (±SD) urinary excretion of 3-hydroxyisovaleric acid (3HIA), biotin, bisnorbiotin (BNB), and biotin sulfoxides (BSOs) in 11 subjects from study day 0 to study day 28. Subjects consumed an egg white beverage with each meal to produce marginal biotin deficiency. NR, normal range. *Significantly different from study day 0 at the level indicated in each panel by post hoc test after ANOVA.

If overall trends were significant at $P < 0.05$, Fisher's post hoc test with Bonferroni correction (32) was used to define the time points that were significantly different from study day 0. The P values given in the figures are the least significant value between any 2 time points and are not less than the overall significance of the ANOVA itself. The significance of trends with duration of repletion was tested by two-way ANOVA with repeated measures; the within factor was time and the between factor was supplement group (immediate versus late supplement). If trends with time were significant ($P < 0.05$), Fisher's post hoc test with Bonferroni correction was used to define the time points that were significantly different from study day 28. If the interaction of group with time was significant, treatment subgroups were analyzed separately. If the interaction was not significant, immediate and late groups were pooled before post hoc testing of the time points. STATVIEW 5.01 (SAS Institute, Cary, NC) was used for the analyses.

RESULTS

Effects of loading and washout

Neither the mean urinary excretion of biotin (50 ± 27 nmol/d) nor the mean urinary excretion of 3HIA (98 ± 31 μmol/d) on study day 0 was significantly different from that of a group studied previously without a load and washout (49 ± 31 nmol/d and 113 ± 34 μmol/d, respectively). Thus, these indicators suggest that initial biotin status was not altered by loading and washout.

Signs and symptoms of biotin deficiency

There was a significant ($P = 0.026$ by paired means comparison) but biologically unimportant weight loss; the mean (±SD) body weight difference for study day 28 compared with study day 0 was −0.69 ± 0.88 kg. Each subject was closely monitored by the GCRC staff for characteristic signs of biotin deficiency such as hair loss and skin rash. No subject developed such symptoms. One subject reported an evanescent red rash on study day 28; the rash was not apparent to an examiner at discharge (study day 28) and did not recur. About one-half of the subjects noticed an unusual body odor or were reported by roommates or staff to have an unusual body odor; to our knowledge, body odor has not been reported previously as a consequence of biotin deficiency.

Biotin depletion

The mean urinary excretion rates of 3HIA, biotin, bisnorbiotin, and biotin sulfoxides for the 11 subjects over the 28 d of egg white feeding are shown in **Figure 1**. Excretion of 3HIA increased steadily and significantly ($P < 0.0001$). Compared with study day 0, the mean increase was significant by study day 17. Excretion of 3HIA was greater than the upper limit of normal for 3 subjects by study day 7, for 8 subjects by study day 14, and for 9 subjects by study day 28.

Biotin excretion decreased rapidly and significantly during depletion ($P < 0.0001$). Compared with study day 0, the mean decrease was significant by study day 7. For all subjects, the mean biotin excretion on study day 28 was 22% of the value on study day 0. On study day 14, biotin excretion was less than normal for 8 of the 11 subjects; on study day 28, biotin excretion was less than the lower limit of normal for 9 of the 11 subjects.

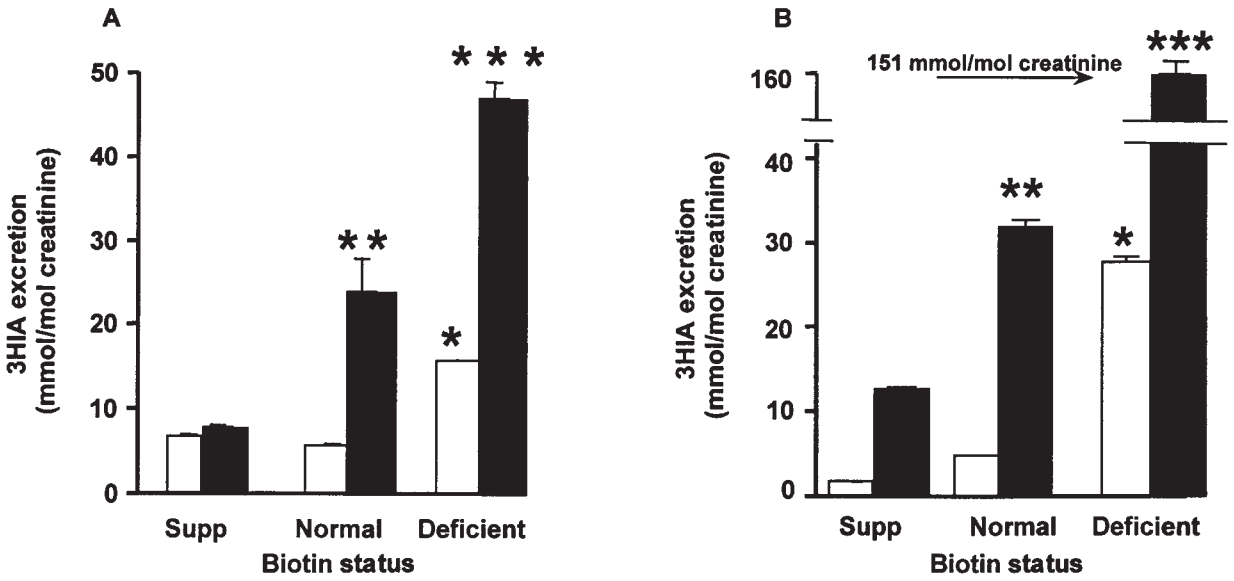

FIGURE 2. 3-Hydroxyisovaleric acid (3HIA) response to a leucine challenge: dose selection study. □, baseline 3HIA excretion; ■, challenge 3HIA excretion; Supp, supplemented. Error bars denote ± 1 SD of analytic replicates ($n \geq 3$ replicates). A: Dose used was 35 mg leucine/kg body wt; $n = 1$ subject per biotin status category. Challenge 3HIA excretion increased with decreasing biotin status. Baseline 3HIA excretion also increased with decreasing biotin status. *Significantly different from supplemented and normal, $P < 0.0003$. **Significantly different from supplemented and deficient, $P < 0.0003$. ***Significantly different from supplemented, $P < 0.0003$. B: Dose used was 70 mg leucine/kg body wt. Challenge 3HIA excretion was related to biotin status, such that supplemented < normal < deficient. Baseline excretion also increased with decreasing biotin status.*Significantly different from supplemented and normal, $P < 0.0003$. **Significantly different from supplemented and deficient, $P < 0.001$. ***Significantly different from supplemented, $P < 0.0001$.

Before egg white feeding, the bisnorbiotin excretion rates of the subjects were not significantly different from the group used to determine the normal range. No subject excreted less bisnorbiotin than the lower limit of normal. However, 4 subjects excreted more bisnorbiotin than the upper limit of normal. Bisnorbiotin excretion decreased rapidly and significantly during depletion ($P < 0.0001$). Compared with study day 0, the mean decrease was significant by study day 3. For all subjects, the mean bisnorbiotin excretion on study day 28 was 9% of the value at study day 0. On study day 14, bisnorbiotin excretion was less than normal for 7 of the 11 subjects; on study day 28, bisnorbiotin excretion was less than the lower limit of normal for 10 of the 11 subjects.

Before egg white feeding, the mean excretion rate of biotin sulfoxides of the study subjects was not significantly different from normal. One subject excreted less biotin sulfoxides than the lower limit of normal, and 6 subjects excreted more biotin sulfoxides than the upper limit of normal. Biotin sulfoxide excretion decreased significantly with depletion ($P < 0.0001$); excretion of biotin sulfoxides decreased by ≈50% during the first week of depletion and changed slowly thereafter. Compared with study day 0, the decrease was significant by study day 3. For all subjects, the mean biotin sulfoxide excretion on study day 28 was 9% of the value at study day 0. On study day 14, biotin sulfoxide excretion was less than normal for 6 of the 11 subjects; on study day 28, biotin sulfoxide excretion was less than the lower limit of normal for 7 of the 11 subjects.

We sought to determine whether either the urinary excretion of 3HIA or the urinary excretion of biotin differed between the sexes. We concluded that pooling of the data from the current study ($n = 3$ men and 8 women) and the study reported in 1997 ($n = 8$ men and 2 women; 10) was justified despite differences in study design such as the loading and washout period in the current study because initial biotin status was not significantly different between the 2 studies. Before egg white feeding, 3HIA excretion was not significantly different between men ($\bar{x}$ ± SD: 114 ± 36 μmol/d; $n = 10$) and women (98 ± 30 μmol/d; $n = 11$). Nor was biotin excretion significantly different between men (58 ± 36 nmol/d) and women (42 ± 18 nmol/d).

We also sought to determine whether susceptibility to biotin depletion differed between the sexes. We used the pooled data on the urinary excretion of 3HIA and biotin on study day 21 from the 1997 study and from the current study. Mean 3HIA excretion was not significantly different between men (412 ± 197 μmol/d) and women (282 ± 212 μmol/d). Likewise, biotin excretion did not differ significantly between the sexes (17 ± 11 for men and 13 ± 5 nmol/d for women).

Preliminary leucine dose study

The dose used for the leucine challenge in the current study was determined in a preliminary dose-ranging study that included 2 doses of leucine. We initially evaluated 35 mg leucine/kg body wt because this dose is one-half of the average daily intake. We observed that the highest rate of 3HIA excretion varied among the individuals (data not shown); the increased excretion over baseline was largely complete by 6 h after ingestion of the leucine. On the basis of the excretion rates observed in the 2-h collections, a collection interval of 5 h was selected for the standard leucine challenge; all voids during this interval were pooled. Results were expressed as mmol 3HIA excreted/mol creatinine in the pooled urine collection. As shown in **Figure 2**, both baseline 3HIA excretion and challenge 3HIA excretion increased significantly ($P < 0.0001$ by one-way ANOVA). Single individuals were tested

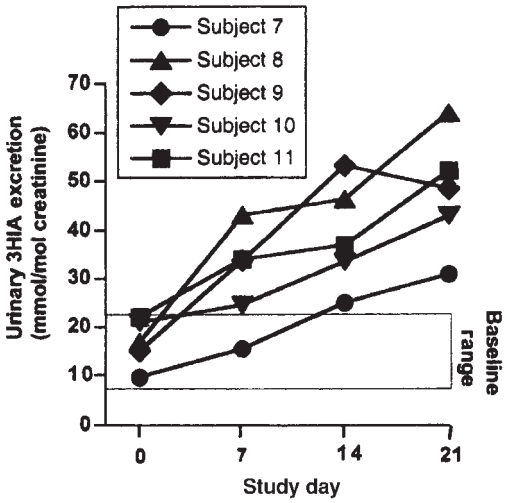

FIGURE 3. Leucine challenge. Urinary excretion of 3-hydroxyisovaleric acid (3HIA) in 5 subjects (2 women) who underwent an oral leucine challenge on study days 0, 7, 14, and 21. Bars representing analytic variability were approximately the size of the symbols and are not shown. The leucine dose of 70 mg/kg body wt was taken in the morning; all urine was collected for the next 5 h. The clear box represents the range of excretion at study day 0.

at each dose and biotin status; thus, we could not infer whether differences were attributable to biotin status or individual differences. For the purposes of designing the leucine challenge, we assumed that the differences arose from biotin status rather than individual differences.

We next evaluated 70 mg/kg (Figure 2). Greater challenge 3HIA excretion rates were observed with the larger dose, and challenge 3HIA excretion rates increased significantly as biotin status decreased ($P < 0.0001$); no adverse symptoms were elicited. This dose was chosen for the current study.

Leucine challenge during depletion

The final 5 subjects participating in the main depletion and repletion study underwent a 70-mg/kg leucine challenge on study days 0, 7, 14, and 21. The mean challenge excretion rate for the group of 5 subjects increased significantly with time on the egg white diet ($P < 0.002$ by one-way ANOVA). By study day 7, the challenge 3HIA excretion rates for 4 of the 5 subjects were greater than the greatest challenge 3HIA excretion rate on day 0 (**Figure 3**). On study days 14 and 21, the values for all 5 subjects were greater than the baseline range.

Repletion phase

The mean urinary excretion rates of 3HIA, biotin, bisnorbiotin, and biotin sulfoxides during 21 d of biotin repletion are shown in **Figure 4**. 3HIA excretion decreased dramatically and significantly ($P < 0.0001$). Excretion of 3HIA was not significantly different between those who started an 80 μg biotin supplement on day 28 (immediate supplement group; $n = 5$) and those who consumed only a mixed general diet until day 35 (late supplement group; $n = 6$). The interaction between time and supplementation group was also not significant. For the pool of all subjects, excretion on both day 35 and day 49 was significantly less than that on day 28 ($P < 0.0002$). By study day 49, the 3HIA excretion rates of all 11 subjects were normal.

For biotin excretion, two-way ANOVA with repeated measures was significant for time ($P < 0.0001$), supplement group ($P = 0.02$), and for the interaction ($P = 0.03$). For the immediate supplement group, biotin excretion increased dramatically and significantly ($P < 0.003$) from study day 28 to 35. A further modest increase from study day 35 to study day 49 was observed, but the increase was not significant. For the late supplement group, biotin excretion did not increase significantly from study day 28 to 35. A substantial and significant increase occurred in response to the later initiation of biotin supplementation ($P < 0.003$ for study day 49 compared with study day 0). In the immediate supplement group, biotin excretion returned to normal for 5 of 5 subjects by study day 35. Biotin excretion of one subject (no. 9) in this group had returned to normal by study day 35, then fell to below the normal range by study day 49 despite 14 d of supplementation, raising the question of compliance with the biotin supplementation regimen as an outpatient. In the late supplement group, biotin excretion for 1 of 6 subjects remained abnormal at study day 35, but was normal by study day 49.

In both groups, the response of bisnorbiotin excretion to repletion was similar to that of biotin and was significant ($P < 0.0001$). Neither the difference between supplement groups nor the interaction was significant. In the pooled group of 11 subjects, the increases from study day 0 to study day 35 and to study day 49 were both significant ($P < 0.0001$). Subject no. 9 also excreted abnormally low bisnorbiotin at study day 49.

The response of biotin sulfoxide excretion to repletion was similar to that of biotin and was significant ($P < 0.0002$). Neither the difference between supplement groups nor the interaction was significant. In the pooled group of 11 subjects, the increases from study day 0 to study day 35 and to study day 49 were significant ($P < 0.0004$). The biotin sulfoxide excretion of subject no. 9 also decreased dramatically from study day 35 to study day 49.

DISCUSSION

The results of clinical studies have provided evidence that marginal biotin deficiency is more common than previously thought. For example, our recent studies provide evidence that marginal, asymptomatic biotin deficiency is common in normal human pregnancy (1, 2). To cite another example, Velazquez (3) reported laboratory evidence of biotin deficiency and biotin-responsive rashes in children with severe protein-energy malnutrition. He speculated that biotin deficiency might be rate limiting in the nutritional rehabilitation of these patients. To cite a third example in both children and adults, marginal biotin deficiency appears to be a frequent consequence of long-term therapy with certain anticonvulsants (4–8).

Properly diagnosing marginal biotin deficiency is important because marginal deficiency may have deleterious health effects. For example, marginal biotin deficiency may be teratogenic (33). In all those studies, assertions concerning the presence of biotin deficiency depended on the validity of indicators of biotin status. The findings of this study provide confirmation that the urinary excretion of biotin and the urinary excretion of 3HIA are early and sensitive indicators of biotin deficiency. These studies also provide evidence that resumption of a mixed general diet repletes biotin status within 1 wk in most individuals as judged both by urinary biotin excretion and 3HIA excretion. Moreover, the rate of repletion is accelerated by supplementation with 80 μg (328 nmol) biotin/d.

In this study, the urinary excretion rates of biotin and bisnorbiotin were abnormally decreased in one individual despite 7 d of a

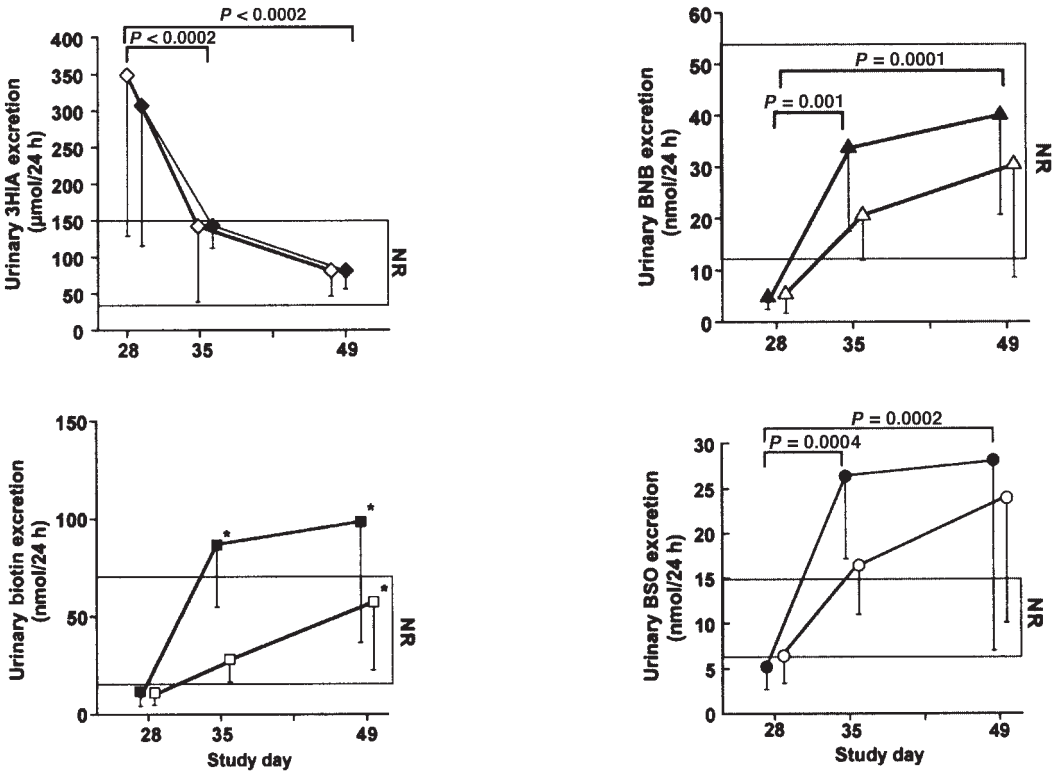

FIGURE 4. Repletion phase. Mean (± SD) urinary excretion of 3-hydroxyisovaleric acid (3HIA), biotin, bisnorbiotin (BNB), and biotin sulfoxides (BSOs) for the immediate supplement (filled symbols; $n = 5$) and late supplement (open symbols; $n = 6$) groups at study days 28, 35, and 49. Time points have been offset slightly for graphic clarity. NR, normal range. Because neither supplement group nor the interaction of supplement group with time was significantly different for 3HIA, BNB, or BSO, for these indicators of biotin status, testing of trends with time was performed on the pooled subjects ($n = 11$); P values are provided on bars. For biotin, both the effect of supplement group and the interaction of supplement group with time were significant ($P = 0.02$ and $P = 0.03$, respectively); statistical comparisons are for supplement groups considered separately. *Significantly different from study day 28, $P < 0.001$ (post hoc test after ANOVA).

mixed general diet followed by 14 d of biotin supplementation. This subject's 3HIA excretion returned to normal after 1 wk on a general diet and remained normal during biotin supplementation. This subject was 1 of the 2 individuals whose biotin excretion on day 0 was less than the lower limit of normal, although 3HIA excretion was normal. This may represent population variation, although failure to take the biotin supplement is also a possibility. Thus, these 2 indicators conflicted concerning this subject's biotin status. The urinary 3HIA excretion of a different individual remained well within the normal range for the first 24 d of egg white feeding, rising to twice the upper limit of normal on the last day of the biotin depletion. Yet, her excretion of biotin and bisnorbiotin was abnormally low after study day 14. We speculate that this individual was indeed becoming biotin deficient and that she represents a variant of normal in which the byproducts of the buildup of methylcrotonyl CoA are not shunted primarily into 3HIA. Further studies of this individual are underway. Results from these 2 subjects exemplify our growing impression that neither of these 2 indicators used alone is sufficient to identify every individual who is marginally deficient.

Both of these validated indicators depend on renal function. Development of a valid indicator that does not depend on renal function would likely be useful. Unfortunately, the plasma concentration of biotin is not particularly useful in detecting marginal biotin deficiency. Moreover, the plasma concentration of biotin is quite elevated in the first trimester of pregnancy for unknown reasons (2); the increase is not attributable to increased protein binding (2). Likewise, the concentration of biotin in erythrocytes is similar to the plasma concentration for the same blood sample (unpublished data); this equivalency likely depends on time for biotin to equilibrate across the erythrocyte membrane, either in vivo or in vitro. Studies of propionyl-CoA carboxylase (EC 4.4.4.41) in lymphocytes of biotin deficient patients receiving parenteral nutrition (34) suggest this enzyme activity may be a more promising indicator for future studies.

One approach to comparing the sensitivity of several indicators in detecting marginal biotin deficiency is to compare the proportion of individuals who are identified as deficient at various points during progressive deficiency. For example, by study day 14, biotin excretion was less than the lower limit of normal for 8 of the 11 subjects and bisnorbiotin excretion was less than the lower limit of normal for 7. At this same point in the progression of biotin deficiency, the urinary excretion of 3HIA was increased in 8 subjects. Thus, it appears that biotin, bisnorbiotin, and 3HIA have approximately equal efficacy in detecting marginal biotin deficiency.

If the normal range is defined as the smallest and largest challenge 3HIA excretion rates for the 5 subjects on study day 0, then 4 of the 5 would be identified as biotin deficient by study day 7 and all 5 thereafter. For comparison, biotin excretion was less than the lower limit of normal in 4 and bisnorbiotin was less than the

lower limit of normal in 2 of the 11 subjects on study day 7; 3HIA excretion was greater than the upper limit of normal in 3 of the 11 subjects on study day 7. By these criteria, the urinary excretion of 3HIA in response to a leucine challenge is a potentially useful index of marginal biotin deficiency. The strength of this conclusion is currently limited by the small number of subjects studied.

A variety of lines of evidence indicate that increased urinary excretion of 3HIA is the result of accumulation of methylcrotonyl CoA (the substrate for methylcrotonyl-CoA carboxylase) or its immediate precursor isovaleryl CoA. The observed progressive increases in 3HIA excretion after a leucine challenge during progressive biotin deficiency are consistent with this mechanism. The blunted responses to leucine challenge observed in biotin-supplemented subjects compared with that in subjects with normal biotin status suggest that methylcrotonyl-CoA carboxylase is close to rate limiting in the leucine degradation pathway, even in persons with normal biotin status. Thus, the observation that 24-h excretion is an early indicator of biotin depletion is consistent with the leucine challenge results.

To assess whether supplementation at the adequate intake would have a significant effect on accelerating repletion, we provided a commercially available biotin supplement with a labeled content of 30 μg during the repletion phase. Although our previous measurement of the biotin content of a broad range of commercially available vitamins compounded from pure sources indicated that the content was accurate (17), the assayed content of the chosen supplement was 80 ± 13 μg. Thus, the results of this study indicate that supplementation at roughly 3 times the adequate intake (16) augments the urinary excretion of biotin to above the normal range in most patients, but the data from this study do not address the effect of the supplement at the adequate intake.

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REFERENCES

1. Mock DM, Stadler DD. Conflicting indicators of biotin status from a cross-sectional study of normal pregnancy. J Am Coll Nutr 1997; 16:252–7.
2. Mock DM, Stadler D, Stratton S, Mock NI. Biotin status assessed longitudinally in pregnant women. J Nutr 1997;127:710–6.
3. Velazquez A, Martin-del-Campo C, Baez A, et al. Biotin deficiency in protein-energy malnutrition. Eur J Clin Nutr 1988;43:169–73.
4. Krause K-H, Berlit P, Bonjour J-P. Impaired biotin status in anticonvulsant therapy. Ann Neurol 1982;12:485–6.
5. Krause K-H, Berlit P, Bonjour J-P. Vitamin status in patients on chronic anticonvulsant therapy. Int J Vitam Nutr Res 1982;52:375–85.
6. Krause K-H, Kochen W, Berlit P, Bonjour J-P. Excretion of organic acids associated with biotin deficiency in chronic anticonvulsant therapy. Int J Vitam Nutr Res 1984;54:217–22.
7. Mock DM, Dyken ME. Biotin catabolism is accelerated in adults receiving long-term therapy with anticonvulsants. Neurology 1997; 49:1444–7.
8. Mock DM, Mock NI, Lombard KA, Nelson RP. Disturbances in biotin metabolism in children undergoing long-term anticonvulsant therapy. J Pediatr Gastroenterol Nutr 1998;26:245–50.
9. Said HM. Biotin bioavailability and estimated average requirement: why bother? Am J Clin Nutr 1999;69:352–3 (editorial).
10. Mock N, Malik M, Stumbo P, Bishop W, Mock D. Increased urinary excretion of 3-hydroxyisovaleric acid and decreased urinary excretion of biotin are sensitive early indicators of decreased status in experimental biotin deficiency. Am J Clin Nutr 1997;65:951–8.
11. Hardinge MG, Crooks H. Lesser known vitamins in food. J Am Diet Assoc 1961;38:240–5.
12. Hoppner K, Lampi B, Smith DC. An appraisal of the daily intakes of vitamin B_{12}, pantothenic acid and biotin from a composite Canadian diet. Can Inst Food Sci Technol J 1978;11:71–4.
13. Bull NL, Buss DH. Biotin, pantothenic acid and vitamin E in the British household food supply. Hum Nutr Appl Nutr 1982;36A: 125–9.
14. Lewis J, Buss DH. Trace nutrients: minerals and vitamins in the British household food supply. Br J Nutr 1988;60:413–24.
15. Zempleni J, Mock DM. Biotin biochemistry and human requirements. J Nutr Biochem 1999;10:128–38.
16. National Research Council. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B-6, folate, vitamin B-12, pantothenic acid, biotin, and choline. In: Food and Nutrition Board, Institute of Medicine, ed. Recommended dietary allowances. 11th ed. Washington, DC: National Academy Press, 1998:415.
17. Tolaymat N, Mock DM. Biotin analysis of commercial vitamin and other nutritional supplements. J Nutr 1989;119:1357–60.
18. Pennington JAT. Biotin. Bowes and Church's food values of portions commonly used. 15th ed. Philadelphia: JP Lippincott Co, 1989.
19. Pennington JAT, Church HN. Biotin. Bowes and Church's food values of portions commonly used. 14th ed. New York: Harper & Row, 1985.
20. Green NM, Toms EJ. Purification and crystallization of avidin. Biochem J 1970;118:67–70.
21. Green NM. Avidin. Adv Protein Chem 1975;29:85–133.
22. Zeman FJ. Clinical nutrition and dietetics. 2nd ed. New York: Macmillan Publishing, 1991.
23. Mock DM, Malik MI. Distribution of biotin in human plasma: most of the biotin is not bound to protein. Am J Clin Nutr 1992;56:427–32.
24. Mock DM, Lankford GL, Cazin Jr J. Biotin and biotin analogs in human urine: biotin accounts for only half of the total. J Nutr 1993; 123:1844–51.
25. Zempleni J, McCormick DB, Mock DM. Identification of biotin sulfone, bisnorbiotin methyl ketone, and tetranorbiotin-*l*-sulfoxide in human urine. Am J Clin Nutr 1997;65:508–11.
26. Mock DM, Mock NI. Serum concentrations of bisnorbiotin and biotin sulfoxide increase during both acute and chronic biotin supplementation. J Lab Clin Med 1997;129:384–8.
27. Mock DM, Heird GM. Urinary biotin analogs increase in humans during chronic supplementation: the analogs are biotin metabolites. Am J Physiol Endocrinol Metab 1997;272:83–7.
28. Mock DM. Determinations of biotin in biological fluids. In: McCormick DB, Suttie JW, Wagner C, eds. Methods in enzymology. New York: Academic Press, 1997:265–75.
29. Mock DM, Lankford GL, Mock NI. Biotin accounts for only half of the total avidin-binding substances in human serum. J Nutr 1995;125:941–6.
30. Heingard D, Tiderstrom G. Determination of serum creatinine by a direct calorimetric method. Clin Chim Acta 1973;43:305–10.
31. Rose DB. Clinical physiology of acid-base and electrolyte disorders. In: Jeffers J, Navrozov M, eds. Renal physiology. New York: McGraw-Hill, Inc, 1994:44–65.
32. Zar JH. Biostatistical analysis. Englewood Cliffs, NJ: Prentice-Hall, Inc, 1974.
33. Zempleni J, Mock DM. Marginal biotin deficiency is teratogenic. Proc Soc Exp Biol Med 2000;223:14–21.
34. Velazquez A, Zamudio S, Baez A, Murguia-Corral R, Rangel-Peniche B, Carrasco A. Indicators of biotin status: a study of patients on prolonged total parenteral nutrition. Eur J Clin Nutr 1990;44:11–6.