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Lymphocyte propionyl-CoA carboxylase and accumulation of odd-chain fatty acid in plasma and erythrocytes are useful indicators of marginal biotin deficiency☆

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Abstract

Background: Recent studies indicate marginal biotin deficiency is more common than previously thought. That conclusion's validity rests on two indicators of biotin status that depend on renal function.

Objective: Assessing the validity of two indicators of biotin status that do not depend upon renal function: 1) activity of the biotin-dependent enzyme propionyl-CoA carboxylase (PCC) in lymphocytes and 2) accumulation of odd-chain fatty acids in the lipids of plasma and erythrocytes.

Design: Marginal biotin deficiency was induced in 11 healthy adults by egg-white feeding for 28 days. Blood and 24-h urine samples were collected before commencing the diet and twice weekly thereafter. After depletion, biotin status was restored with a general diet with or without 80 μg/day or 328 nmol/day biotin supplement. Activity of PCC was determined by an optimized NaH $^{14}CO_3$ incorporation assay. Fatty acid composition was determined by gas chromatography.

Results: With time on the egg-white diet, lymphocyte PCC activity decreased significantly ($P < 0.0001$); C15:0 and C17:0 content increased significantly in the lipids of plasma and erythrocytes ($P < 0.015$). In eight of 11 subjects, lymphocyte PCC activity returned to normal within three weeks of resuming general diets with or without biotin supplement. With repletion, C15:0 and C17:0 in plasma lipids decreased ($P < 0.02$), but odd-chain content of erythrocytes did not decrease significantly.

Conclusions: Lymphocyte PCC activity is an early and sensitive indicator of marginal biotin deficiency. Odd-chain fatty acids accumulate in blood lipids more gradually during marginal deficiency and return to normal more gradually after biotin repletion. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Biotin deficiency; propionyl-CoA carboxylase; odd-chain fatty acids; humans; plasma; erythrocytes

1. Introduction

Recent clinical studies from 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. We define marginal biotin deficiency as a condition where tissue biotin status has been depleted to the extent that, though there may not be signs or symptoms of frank biotin deficiency, there are changes in certain biochemical parameters that reveal decreases in normal biotin status. As noted in a recent editorial, there is a need to develop valid indicators of marginal biotin deficiency [9]. Our preliminary studies provide evidence that biotin status does not correlate well with biotin intake, at least in children. Accordingly, we tentatively conclude that the usefulness of terminology referring to biotin intake is limited in regard to the current study.

We recently reported the results of the first experimental

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study to successfully induce biotin deficiency in human subjects since the 1940's. That study provided evidence that decreased urinary excretion of biotin and increased urinary excretion of 3-hydroxyisovaleric acid (3HIA) are early and sensitive indicators of reduced biotin status. Biotin is a covalently bound prosthetic group for four mammalian carboxylases; one of these, methylcrotonyl-CoA carboxylase, catalyzes an essential step in the intermediary metabolism of 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.

Development of a valid indicator of biotin status 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 [10] and perhaps not even in detecting moderately severe biotin deficiency [11]. The concentration of biotin in erythrocytes is similar to the plasma concentration in the same blood sample (unpublished data) and thus is not likely to be useful for detecting marginal biotin deficiency.

Propionyl-CoA carboxylase (PCC) also requires biotin as a cofactor. This enzyme catalyzes the conversion of propionyl CoA to methylmalonyl CoA. This enzyme catalyzes an essential step in the intermediary metabolism of several essential amino acids, odd-chain fatty acids, and the side chain of cholesterol. Studies of PCC activity in lymphocytes of biotin deficient patients receiving parenteral nutrition [12] or suffering from protein-energy malnutrition [11] suggest this enzyme activity might reflect biotin status. In this study, we sought to determine whether PCC in lymphocytes can detect marginal biotin deficiency in healthy adults rendered marginally biotin deficient by egg-white feeding.

Severe biotin deficiency is associated with increases in the percentages of odd-chain fatty acids [13–15]. The mechanism for the accumulation of odd-chain fatty acids is thought to be the abnormal substitution of propionyl CoA (3 carbon) for acetyl CoA (2 carbon) as the "primer" for the first step in the synthesis of long-chain fatty acids by acetyl-CoA carboxylase. In this study, we also investigated whether odd-chain fatty acid accumulation was an indicator of marginal biotin deficiency.

2. Experimental Procedures

2.1. 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 normal adult volunteers (ten women) enrolled in a twenty-eight day study while residing in a general clinical research center (GCRC). Subjects were allowed to leave the GCRC during weekdays, taking their noon meal and urine collection vessels with them; otherwise, subjects resided full-time at the GCRC.

Individuals who were supplementing with dietary biotin deliberately or inadvertently 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 to the estimated daily dietary intake of 35–70 μg/day (143–287 nmol/day) [16–20]. The estimates of biotin content are not precise, 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. Thus, potential volunteers taking any type of supplement were excluded unless negligible biotin content was confirmed by assay. Eleven subjects (eight women) completed the study. The four 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.

2.2. Study design

2.2.1. 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). The content of the supplement was confirmed by assay and was within 10% of the reported value. This supplement is ten times the recommended adequate intake of 30 μg. This loading phase was intended to ensure that all subjects began the egg-white diet with similar biotin nutritional status. In addition, a daily vitamin supplement not containing biotin was given. This supplement contained vitamin A (5000 IU); vitamin C (60 mg, 340 μmol); vitamin D (400 IU); vitamin E (15 IU); thiamin (1.5 mg, 5 μmol); riboflavin (1.7 mg, 5 μmol); niacin (20 mg, 163 μmol); vitamin B6 (2 mg, 10 μmol); folate (400 μg, 1 μmol); vitamin B12 (6 μg, 4 nmol); and iron (18 mg, 322 μmol).

On Study Day -7, the biotin supplement was discontinued beginning 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 using the avidin-binding assay [21]. 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 less than 0.09% of the estimated daily dietary intake of 35–70 μg/day (143–287 nmol/day) [16,17] and 0.3% of the study dietary intake of 10.2 μg (42 nmol) for women and 13.8 μg (56 nmol) for men.

2.2.2. Biotin depletion

On Study Day –1, subjects were admitted to the GCRC. On Study Day 0, subjects provided a 30 ml blood sample. Blood samples were then collected twice weekly for four weeks and weekly for two additional weeks. Upon collec-

Table 1 Calculated nutrient composition of the two diets

Nutrient	Amount per 10 MJ	
	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 (μmol)	191	277
Biotin (nmol)	94	56

tion, all blood samples were placed in heparinized tubes. Plasma and erythrocyte aliquots were stored at -80°C until analysis. Sucrose buffer and detergent were added to lymphocyte aliquots and the tubes were stored at -80°C until analysis.

Feeding of the research diet (referred to hereafter as the egg-white diet) commenced on Study Day 0. Consumption of undenatured (raw or freeze dried) egg white will prevent absorption of dietary biotin and any biotin released by intestinal biotin. The active biotin-binding protein in egg white is avidin, which is resistant to proteolysis unless denatured. The egg white was provided as dried albumin (Wakefield Brand, M.G. Waldbaum, Wakefield, NE) in a blended beverage containing 16 g of dried egg white per MJ of dietary energy; the egg white accounted for 22% to 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 about 78 times the dietary biotin intake which was calculated from estimates of biotin in food sources [16,22,23] and measured directly in our laboratory as described below. The calculation used a published value for the avidin content of egg white [24,25] 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 using the Harris Benedict equation [26] to provide a eucaloric menu. The typical daily energy intake was 9,700 kJ for women and 14,000 kJ for men: 23% from protein, 50% from carbohydrate, and 27% from fat; nutrient content was estimated using Nutritionist Five, Version 1.6 (First Data Bank, San Bruno, CA). The typical American diet provides about 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-day rotating menu cycle. To increase variety, the food in each menu was rearranged to produce two 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) of coffee, tea, or diet sodas 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.

2.2.3. Biotin repletion

On Study Day 28, the egg-white diet was discontinued. All subjects consumed a self-selected mixed general diet from Study Day 28 to Study Day 49. Six of the subjects continued taking the multivitamin without added biotin (late supplement group) for the first week of repletion (Study Day 28 to 35). The other five subjects received a daily multivitamin and multimineral supplement with biotin (immediate supplement group). Our intent was to provide a supplement that contained an amount of biotin (30 μg) that was equal to the Adequate Intake (AI). 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% [21]. However, analysis of the multivitamin after beginning the supplement showed that the biotin content was greater than the labeled amount; the content was 80 $\pm$ 13 μg (328 $\pm$ 53 nmol; mean $\pm$ SD; n = 5). The supplement contained the same content of vitamins other than biotin as the biotin-free supplement with the following exceptions: vitamin K (25 μg, 0.1 μmol), vitamin B_6 (2.9 mg, 14 μmol), and pantothenic acid (10 mg, 46 μmol).

From Study Day 35 to 49, all subjects received the 80 μg biotin supplement.

2.3. Analytical methods

2.3.1. Propionyl-CoA carboxylase activity in lymphocytes

Peripheral blood mononuclear cell (PBMC) suspensions were obtained from heparinized blood by density gradient separation. Blood was overlaid onto Histopaque (Sigma, St. Louis, MO) and centrifuged at 400 $\times$ g for 30 min. The buffy coat layer containing the PBMC was removed, resuspended in two volumes of PBS and sedimented at 250 x g for 10 min at room temperature. When PBMC are isolated by this gradient centrifugation procedure, the cell preparation contains almost entirely lymphocytes [27]. In blood, lymphocytes and monocytes are quantitatively the most important mononuclear cells; eosinophils and basophils account for $\leq$ 3% of total PBMC [27]. The monocytes are removed during the process of cell isolation because of their

selective binding to plastic surfaces. In this paper, the term lymphocytes will be used.

The lymphocyte pellet was resuspended in 1 ml of PBS for every 1.5 ml of starting volume of blood. This suspension was aliquoted into 2-ml microfuge tubes for subsequent assays (protein, PCC, etc.) and lymphocytes were pelleted at 3800 x g for 5 min. For the PCC assay, the supernatant was aspirated and the pellet containing about 0.2 mg of protein was resuspended in 90 μl of 25 mmol/L sucrose, 50 mmol/L Tris buffer, pH 7.9, 5 mmol/L reduced glutathione, 1 mmol/L EDTA, and 25 μl of 0.5 g/100 g Triton X-100. The lymphocyte pellets were stored at −80°C for assay of all samples. The lysed lymphocyte pellets were thawed and diluted 1:1 with 90 μl of buffer and 25 μl of 9.2 g/100 g Triton X-100; the final concentration of Triton X-100 = 1 g/100 g. The contents were vortexed vigorously to resuspend and release the membrane-bound enzymes.

The method for determination of lymphocyte PCC activity was as described previously [28] with the following modifications: The reactions were carried out at 30°C for 30 min using 96-well plates. Lymphocyte PCC activity was determined in triplicate using 50 μl of the lymphocyte suspension and 50 μl of the substrate mixture. Background activities were assayed in singlicate. The reaction was stopped by the addition of 25 μl of 8 g/100 ml perchloric acid; 40 μl of reaction mixture was transferred to a 96-well plate containing solid scintillant (LumaPlates, Packard Instruments, Downers Grove, IL). The plates were dried in a hood to remove the unreacted $NaH^{14}CO_3$. The dpm for the ^{14}C-labeled product was determined using a quench curve for ^{14}C in a scintillation counter (Topcount, Packard Instruments). Activity of PCC was normalized by lymphocyte protein measured by BCA protein assay of identical aliquots of lymphocyte pellets suspended in water to avoid interfering substances (Pierce, Rockford, IL).

2.3.2. Fatty acid composition

Erythrocytes and plasma were obtained from the same density gradient preparation that isolated lymphocytes. The erythrocyte membranes were washed to remove hemoglobin. Plasma and membranes were delipidated using the method of Folch [29]. Individual lipid classes were separated by thin-layer chromatography on silica gel 60A thin-layer plates (Whatman K-6, Fisher Scientific, Atlanta, GA) with petroleum ether:ethyl ether:acetic acid (80:20:1, v:v:v) as the developing solvent. Lipid classes were visualized with rhodamine B. Triglycerides and phospholipids were scraped from the plate and methylated as described [29] without elution from the gel. The fatty acid methyl esters were analyzed by gas chromatography (GC) using a Supelcowax-10 column (30 m x 0.25 mm, 0.25 μm film, #24079, Supelco, Bellefonte, PA) in an Hewlett-Packard 5890A gas chromatograph equipped with an HP3365 Chem Station, autosampler, and flame ionization detector. The GC was calibrated using a series of standards, and various fatty acid methyl esters were identified by comparison of retention times to pure standards. Internal standards were not added to the samples; rather the amounts of C15:0 and C17:0 were calculated relative to C16:0 and to C18:0.

2.4. Statistics

The normal range for lymphocyte PCC activity was determined as the minimum and maximum value of the 11 subjects prior to initiation of the egg-white diet. For PCC activity, the significance of the changes from Study Day 0 to Study Day 28 of egg-white feeding (depletion) were tested by one-way ANOVA with repeated measures because data were available for multiple time points. Because the composition of odd-chain fatty acids was expected to (and did) change gradually, odd-chain fatty acid composition was determined only on Study Day 0 and Study Day 28 of the depletion phase. Thus, the significance of changes during the depletion phase were tested by Student's paired one-tailed, t test; a one-tailed test is justified by our expectation that odd-chain fatty acid composition would increase as discussed in the Introduction.

Because PCC activity and odd-chain fatty acid composition were determined at multiple time points during repletion, the significances of changes in these parameters were tested by two-way ANOVA with repeated measures. The "within" factor was Study Day, and the "between" factor was supplement group (immediate vs. late). When a factor was significant at $P < 0.05$, Fisher's post hoc test [30] was used to determine the significance of differences between the time points or supplement groups. P values given are the least significant of the Fisher's comparison P values and are always greater than the overall significance of the ANOVA itself. StatView 5.01 (SAS Institute, Cary, NC) was used for the analyses.

3. Results

3.1. Biotin depletion

3.1.1. Signs and symptoms of biotin deficiency

There was a statistically significant ($P = 0.026$ by paired means comparison), but biologically unimportant weight loss; mean body weight difference for Study Day 28 vs. Study Day 0 was -0.69 ± 0.88 kg (mean ± SD). 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 (#9) 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 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.

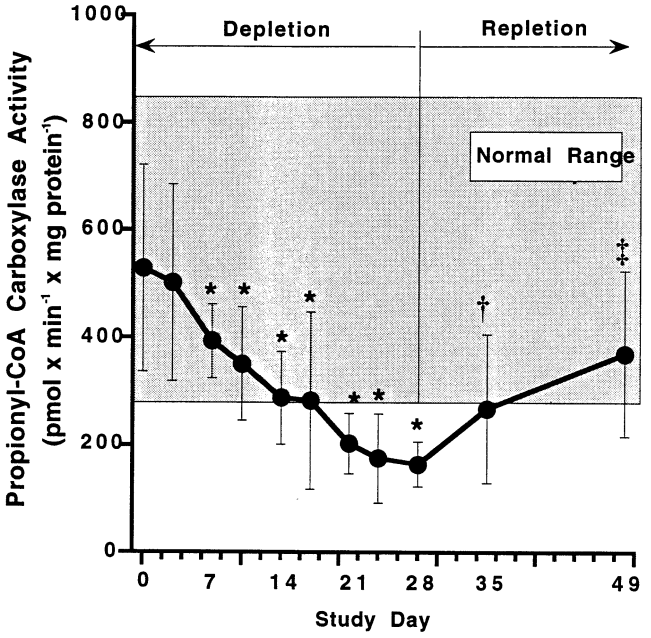

Fig. 1. Effect of biotin depletion and repletion on lymphocyte PCC activity. Eleven subjects consumed an egg-white beverage with each meal from Study Day 0 to Study Day 28 to produce marginal biotin deficiency. After Study Day 28, subjects consumed a mixed diet with or without 328 nmol biotin supplement. Error bars denote ± 1SD. Depletion ANOVA: * denotes Study Day means that are significantly different from Study Day 0 at $P < 0.0004$. Repletion ANOVA: † and ‡ denote Study Day means that are significantly different from Study Day 28 at $P < 0.02$ and $P < 0.0001$, respectively.

3.1.2. Activity of PCC

Lymphocyte PCC activity decreased steadily and significantly ($p < 0.0001$) during the depletion phase of the study (Figure 1). For the 11 subjects, the mean, lymphocyte PCC activity on Study Day 28 was 31% of the value at Study Day 0. The mean decrease was significant by Study Day 7 ($p < 0.01$). On Study Day 14, PCC activity was less than normal for five of the 11 subjects. On Study Day 28, PCC activity was less than the lower limit of normal in all subjects. Results were similar whether expressed per mg of lymphocyte protein (Figure 1) or per mL of blood (data not shown).

3.1.3. Fatty acids

In plasma phospholipids, when normalized by C18:0 content, content of the odd-chain fatty acids C15:0 and C17:0 increased significantly during biotin depletion ($P = 0.0025$ and $P < 0.0001$, respectively) as depicted in Figure 2. For five subjects C15:0/C18:0 content at Study Day 28 was greater than the maximum value of any subject at Study Day 0. For eight of the 11 subjects, the C17:0/C18:0 content increased above the maximum value at Study Day 0. The sum of C15:0 plus C17:0 normalized by C18:0 was also highly significantly increased by Study Day 28; by Study Day 28 the values of seven of 11 subjects were increased above the maximum value at Study Day 0 (data not shown).

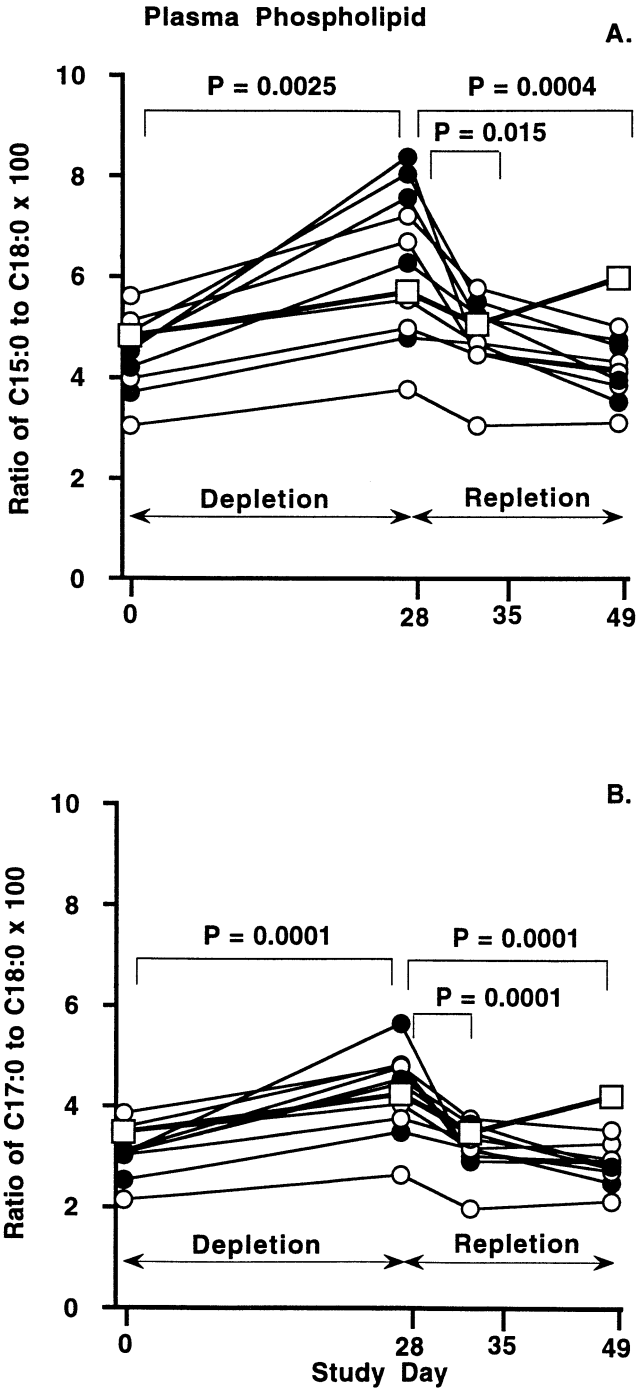

Fig. 2. Effect of biotin deficiency depletion and repletion on odd-chain fatty acid content of plasma phospholipid. Biotin deficiency was induced and repleted as described in Figure 1. After Study Day 28, subjects received a mixed general diet with 328 nmol biotin supplementation beginning on either Study Day 28 (filled symbols ●) or Study Day 35 (open symbols ○). □ Denotes subject #9 who may not have complied with repletion protocol. Panel A. C15:0/C18:0; Panel B. C17:0/C18:0.

Normalizing by C16:0 produced similarly significant changes and a similar diagnostic specificity (data not shown).

For plasma triglycerides, when normalized by C18:0 content, content of the odd-chain fatty acids 15:0 and 17:0

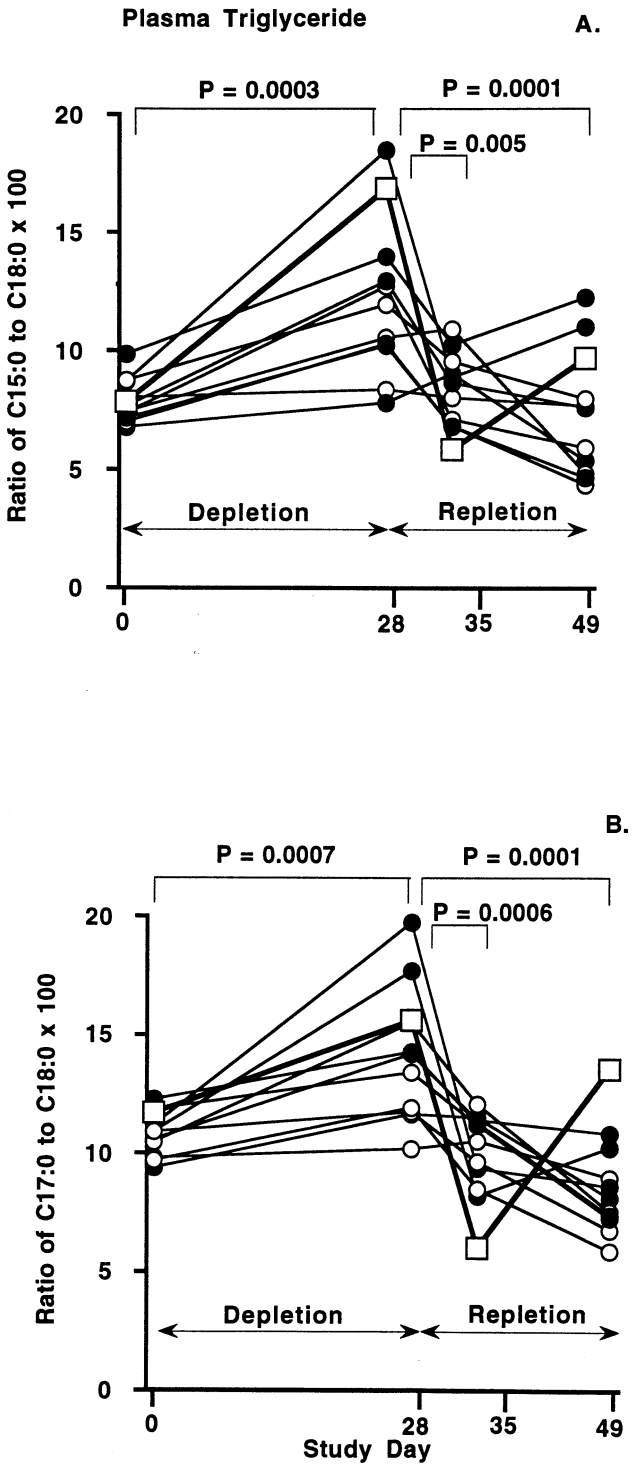

Fig. 3. Effect of biotin deficiency depletion and repletion on odd-chain fatty acid content of plasma triglyceride. Marginal biotin deficiency was induced and repleted as describe in Figure 1. Symbols as per Figure 2. Panel A. C15:0/C18:0; Panel B. C17:0/C18:0.

increased significantly during biotin depletion ($P = 0.0003$ and $P = 0.0007$, respectively) as depicted in Figure 3. For nine of the 11 subjects, the C15:0/C18:0 content at Study Day 28 was greater than the maximum value of any subject at Study Day 0; for seven the C17:0/C18:0 content increased above the maximum value at Study Day 0. The sum of C15:0 plus C17:0 normalized by C18:0 was also highly significantly increased by Study Day 28; by Study Day 28 the values of nine of 11 subjects were increased above the maximum value at Study Day 0 (data not shown). Normalizing by C16:0 produced similarly significant changes and a similar diagnostic specificity (data not shown).

For erythrocyte phospholipids, when normalized by C18:0 content, content of the odd-chain fatty acids 15:0 and 17:0 did not increase during biotin depletion as dramatically as that in plasma lipids, but the change was still significant ($P = 0.02$ and $P = 0.003$, respectively) as depicted in Figure 4. Diagnostic utility was also less. The C15:0/C18:0 content at Study Day 28 was greater than the maximum value of any subject at Study Day 0 for only three subjects. The C17:0/C18:0 content at Study Day 28 was greater than the maximum value at Study Day 0 in seven subjects. The sum of C15:0 plus C17:0 normalized by C18:0 was significantly increased by Study Day 28 ($P < 0.003$; data not shown); by Study Day 28, the values of seven subjects were increased above the maximum value at Study Day 0. Normalizing by C16:0 produced similarly significant changes and a similar diagnostic specificity (data not shown).

3.2. Biotin repletion

3.2.1. Activity of PCC

Mean lymphocyte PCC activities during 21 days of biotin repletion are shown in Figure 1. Activity of PCC increased significantly ($p < 0.0001$) with time, but was not significantly different between the immediate and late supplement groups; the interaction between time and supplement group was not significant. Therefore, supplement groups were pooled for post hoc testing. For the combined group, PCC activity increased significantly from Study Day 28 to Study Day 35 ($P < 0.02$) and from Study Day 28 to Study Day 49 ($P < 0.0001$). By Study Day 35, PCC activity had returned to the normal range for three of the 11 subjects. By Study Day 49, PCC activity had returned to the normal for eight of 11 subjects.

3.2.2. Fatty acids

In response to biotin repletion, plasma phospholipid content of C15:0/C18:0 and of C17:0/C18:0 decreased significantly, but there was not a significant difference between the immediate and late repletion groups. The interaction between time and treatment group was not significant ($P = 0.06$ and $P = 0.08$ respectively) for either C15:0/C18:0 or C17:0/C18:0. For the pooled group of all 11 subjects, the decrease in each odd-chain fatty acid was significant within one week of starting a mixed general diet. Odd-chain fatty acid content decreased further with the additional two weeks of a mixed diet plus biotin supplement (Figure 2). A similar pattern of significantly decreasing content of C15:0/C18:0 and of C17:0/C18:0 was seen in plasma triglycerides (Figure 3).

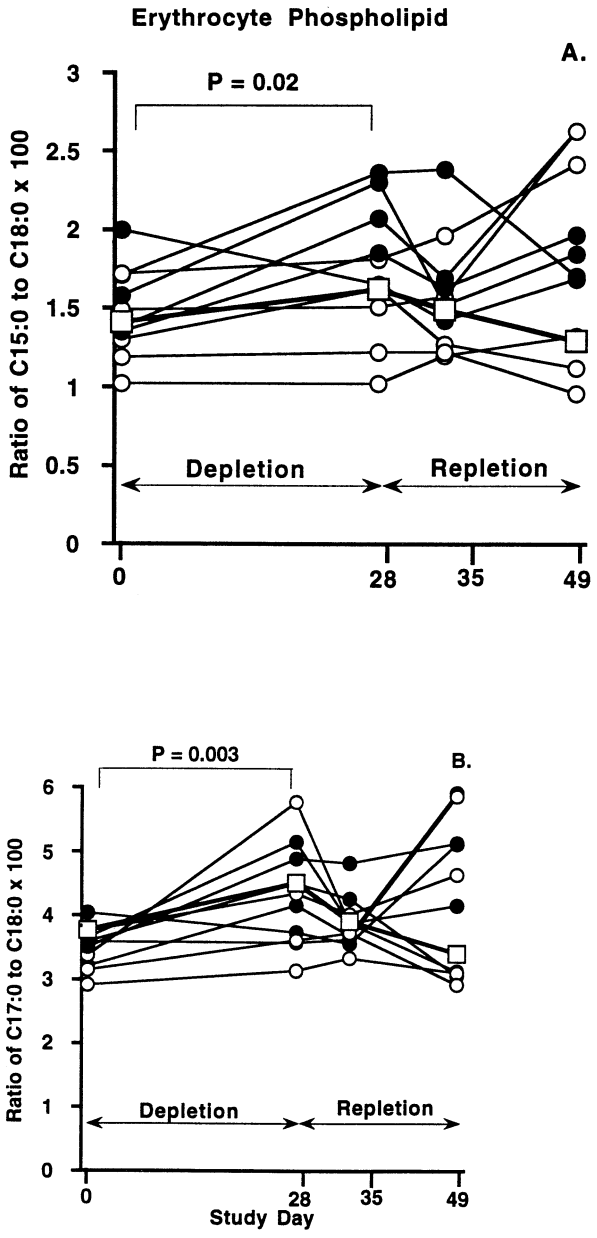

Fig. 4. Effect of biotin deficiency depletion and repletion on odd-chain fatty acid content of erythrocyte membrane phospholipid. Marginal biotin deficiency was induced and repleted as described in Figure 1. Symbols as per Figure 2. Panel A. C15:0/C18:0; Panel B. C17:0/C18:0.

Of particular note was subject #9 who is denoted by θ in Figures 2, 3, and 4. His odd-chain fatty acid content in both plasma phospholipid and plasma triglyceride decreased dramatically on the general diet without biotin supplement, but rebounded dramatically on a mixed diet plus biotin supplement.

In a fashion reminiscent of the less dramatic changes seen in erythrocyte membrane phospholipid during biotin depletion, changes in erythrocyte phospholipid with time of repletion were not significant for either C15:0 or C17:0, nor were there significant differences between the supplement groups (Figure 4). The significance of results changed little, if at all, when fatty acid composition was normalized by C16:0 content rather than C18:0 (data not shown).

4. Discussion

We define marginal biotin deficiency as a condition where tissue biotin status has been depleted to the extent that, though there may not be signs or symptoms of frank biotin deficiency, there are changes in certain biochemical parameters that reveal decreases in normal biotin status. Recent 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 a common occurrence in normal human pregnancy [1,2]. 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 in such situations could be important. For example, marginal biotin deficiency may be teratogenic [31].

In studies of pregnant women and studies of children and adults receiving long-term anticonvulsant therapy, assertions concerning presence of biotin deficiency depended on indicators of biotin status that are affected by renal function. However, renal function is affected by pregnancy. Although the total excretion of creatinine does not change importantly, glomerular filtration rate and creatinine clearance increase with pregnancy [7,32,33]. Thus, development and validation of indicators of biotin status that do not depend on renal function would likely be useful.

In children suffering from severe protein-energy malnutrition, Velazquez reported biotin responsive rashes and evidence of biotin deficiency [3,11]. Of particular interest, the activity of PCC in these children's lymphocytes was decreased and exhibited an increased activation index (i.e., the ratio of enzyme activity in cells incubated with biotin to activity in cells incubated without biotin). In addition, in patients with apparent reduced biotin status due to omission of biotin from prolonged parenteral nutrition, lymphocyte PCC activity was significantly decreased, and increased significantly with biotin repletion [12]. Such findings suggested to us that the activity of lymphocyte PCC might prove to be a good indicator of marginal biotin status.

The studies presented here provide strong evidence that the activity of lymphocytes PCC is an early and sensitive indicator of marginal biotin deficiency. Five of the 11 subjects were identified as deficient by Study Day 14 of egg-white feeding and all were identified by the Study Day 28. To our knowledge the PCC activity at Study Day 28 offers the best specificity of any indicator of biotin status. However, the small number of subjects studied so far limits the strength of this conclusion. Notwithstanding, PCC activity in lymphocytes appears to be both sensitive and specific as an indicator for marginal biotin deficiency. In several animal species, biotin deficiency causes increases in the per-

centages of odd-chain fatty acids [13,14]. In patients who developed severe biotin deficiency during parenteral alimentation, the percentage of C15:0 and C17:0 odd-chain fatty acids in serum increased for each of the four major lipid classes —cholesterol esters, phospholipids, triglycerides, and free fatty acids [15]. The mechanism for the accumulation of odd-chain fatty acids is thought to be the abnormal substitution of propionyl CoA (3 carbon) for acetyl CoA (2 carbon) as the "primer" for the first step in the synthesis of long-chain fatty acid by acetyl-CoA carboxylase. Observations in inborn errors of metabolism that lead to accumulation of propionyl CoA are consistent with this mechanism [34–38]. Based on these observations, we investigated whether odd-chain fatty acid accumulation was an indicator of marginal biotin deficiency.

The studies presented here also provide evidence that odd-chain fatty acids accumulated in the lipids of peripheral blood, particularly plasma phospholipid and triglyceride. Given that fatty acid composition changes relatively slowly (weeks to months) in response to most dietary and metabolic manipulations, the rapidity of the increase and decrease in odd-chain fatty acids could not have been confidently predicted. Although the content of these fatty acids is minute relative to other fatty acid components and the changes are correspondingly small, the response to changes in biotin status seems clear. However, the plasma content of odd-chain fatty acids did not exhibit as great a diagnostic specificity as the decrease in lymphocytes PCC or as the decrease in urinary biotin and increase in urinary 3-hydroxyisovaleric acid observed in previous studies. Notwithstanding, this index of biotin status may offer particular utility in some circumstances because abnormalities are likely to persist several weeks after changes in biotin status. In individuals that are marginally deficient on a chronic basis, larger pools of odd-chain fatty acids will slowly accumulate in organs and then re-equilibrate into plasma phospholipids and triglycerides when biotin status is restored. Thus, this index might be useful in assessing biotin status of pregnant women, individuals receiving chronic therapy with anticonvulsants, and of newborns.

One might question whether the accumulation of odd-chain fatty acids plays a pathogenic role in cutaneous or central nervous system findings of moderate to severe biotin deficiency. However, the studies of Suchy and co-workers [13,39] provide evidence to the contrary with regard to the central nervous system findings. Moreover supplementation with polyunsaturated fatty acids prevented (or at least delayed beyond the end of the experiment) the onset of the cutaneous manifestations of biotin deficiency in rats [28]; this observation suggests that relative sufficiency of fatty acids or subsequent metabolites in the ω6 or ω3 pathways (or both) are more likely the pathogenic factors.

The only subject to develop symptoms potentially related to biotin deficiency was subject #9 who developed a transient rash on the last day of the depletion phase. Although this subject was assigned at random into the late supplement group, we suspect that he may have been noncompliant and actively supplemented biotin based on the response of his PCC data and odd-chain fatty acid composition (Figures 2 and 3) which decreased strikingly during the first week. We also suspect that the subject may not have subsequently complied with his biotin supplementation based on his compliance interview and the subsequent rapid reaccumulation of odd-chain fatty acids between Study Day 35 and Study Day 49. However, we felt that these retrospective observations did not justify excluding the subject data from the statistical analysis. Had the subject been excluded, the result would have been a modest improvement in the statistical significance in the changes in Study Day 35 and Study Day 49 compared to Study Day 28.

On balance, we conclude that the data here indicate that lymphocyte PCC activity is an early and sensitive indicator of marginal biotin deficiency in humans, and that odd-chain fatty acids accumulate more gradually during marginal deficiency and return more gradually to normal after biotin repletion, particularly in erythrocytes. We look forward to applying these kidney-independent functions to investigating biotin status in pregnancy and in the fetus and newborn infant.

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