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Human peripheral blood mononuclear cells: Inhibition of biotin transport by reversible competition with pantothenic acid is quantitatively minor

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A transporter present in intestinal cells and in choriocarcinoma cells has been shown to transport both pantothenic acid and biotin at similar transporter affinities. However, the concentration of pantothenic acid in most foods and biological fluids is approximately 200 times the concentration of biotin; theoretically, pantothenic acid might substantially reduce biotin transport via competition. In the present study, we sought to determine whether pantothenic acid reduces biotin transport by the biotin transporter in peripheral blood mononuclear cells (PBMC). PBMC were isolated from human blood by gradient centrifugation. Incubations with [^{3}H]biotin and pantothenic acid were conducted at physiologic concentrations. Intracellular [^{3}H]biotin was quantified after washing by liquid scintillation counting. Pantothenic acid at 10 to 1,000 nmol/L reduced biotin (475 pmol/L) uptake by less than 12% ($P < 0.05$). Based on Lineweaver-Burk plots, the competition was reversible. Several structural analogs of pantothenic acid at 1,000 nmol/L reduced biotin transport by only 7 to 15% ($P = 0.13$). No pattern of molecular structure required for recognition by the transporter was apparent. Extracellular pantothenic acid did not affect biotin efflux from [^{3}H]biotin-loaded PBMC ($P > 0.05$), suggesting that countertransport of extracellular pantothenic acid and intracellular biotin does not increase biotin efflux from PBMC. We conclude that the physiologic effects of pantothenic acid on the transport of biotin in PBMC are likely to be quantitatively minor. (J. Nutr. Biochem. 10:427–432, 1999)

Keywords: biotin; human; pantothenic acid; peripheral blood mononuclear cells; transport

Introduction

A vitamin transporter that has been documented in epithelial cells transfers both pantothenic acid and biotin. Moreover, in human choriocarcinoma cells,[1] rabbit intestinal cells,[2] and caco-2 cells (a human intestinal malignancy cell line),[2,3] competition between transport of pantothenic acid and biotin has been documented. In addition, pantothenic acid at pharmacologic concentrations inhibited the transport of biotin across the blood-brain barrier.[4] Recently, a single protein responsible for the transport of biotin, pantothenic acid, and lipoic acid across rat placenta has been cloned and functionally expressed.[5] This transporter has been named "the sodium-dependent multivitamin transporter." The affinities of pantothenic acid and biotin for this sodium-dependent vitamin transporter are approximately the same order of magnitude.

Currently, our laboratory is seeking to identify and validate indices of biotin nutritional status in humans. Potentially useful indices include the concentration of biotin in peripheral blood mononuclear cells (PBMC) and activities of biotin-dependent carboxylases in PBMC. If pantothenic acid competes with biotin into PBMC, this could theoretically confound use of PBMC biotin in the assessment of biotin nutritional status. Moreover, some PBMC culture media contain large concentrations of pantothenic acid. For example, RPMI-1640 contains approximately 1 μmol/L pantothenic acid, which is approximately 10 times

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higher than the concentration in normal human plasma.[6] Theoretically, this large concentration of pantothenic acid could interfere with biotin uptake in PBMC transport studies such as those published by our laboratory. In this study, we sought to determine whether pantothenic acid reduces the transport of biotin by the PBMC transporter and, if so, to characterize the mechanism by which pantothenic acid inhibits biotin transport and any structural requirement for the inhibition.

Methods and materials

Materials

[^{3}H]Biotin was purchased from Dupont (Boston, MA USA; specific radioactivity 2.15 TBq/mmol). D-Pantothenic acid, D-pantethine, D-pantothenyl alcohol, D(-)-pantoyllactone, β-alanine, and coenzyme A were purchased from Sigma Chemical Co. (St. Louis, MO USA). To avoid decomposition, pantothenic acid and its analogs were protected against exposure to light.

Subjects

This study was approved by the Human Research Advisory Committee of the University of Arkansas for Medical Sciences (Little Rock, AR USA). Written informed consent was obtained from all subjects.

Peripheral blood for isolation of PBMC was collected from nine Caucasians (four male, five female). The age of the subjects ranged from 20 to 52 years; two of the subjects were smokers. No gender-, age-, or smoking-related differences in biotin nutritional status have yet been identified. None of the subjects consumed vitamin supplements that contained more than the currently recommended adequate intake of biotin (30 μg/d).[7] In previous studies, anticonvulsants have been identified as drugs that may interfere with normal metabolism of biotin.[8–12] None of the subjects in the present study were receiving anticonvulsants.

Isolation of PBMC

For each set of experiments, blood from up to five different subjects was used; blood from individual subjects was not combined. PBMC were isolated from blood by gradient centrifugation as described previously.[13,14] The PBMC pellet from 100 mL of blood was resuspended in 25 mL of phosphate-buffered saline and 5.5 mmol/L D-glucose. Cell density in the suspension was $5.2 \pm 1.4 \times 10^6$ cells/mL. Viability of PBMC was measured by exclusion of 4.16 mmol/L trypan blue; viability was 99.4 ± 0.6% for freshly isolated cells and 98.7 ± 1.0% at the end of the experimental sessions ($P < 0.05$, as determined by paired, two-tailed *t*-test).

Quantitation of [^{3}H]biotin uptake and efflux

One milliliter aliquots of PBMC suspensions were incubated with [^{3}H]biotin and either D-pantothenic acid, D-pantethine, D-pantothenyl alcohol, D(-)-pantoyllactone, β-alanine, or coenzyme A at 37°C for 30 minutes as previously described.[14] Concentrations of [^{3}H]biotin, pantothenic acid, and pantothenic acid analogs are provided with each experiment in the Results section. Intracellular [^{3}H]biotin was quantitated by sedimentation of PBMC, washing away the extracellular medium, and liquid scintillation counting as described previously.[14] We have shown previously that PBMC do not catabolize biotin; hence, [^{3}H] in PBMC quantitates [^{3}H]biotin rather than [^{3}H]biotin plus [^{3}H]biotin metabolites.[14]

[^{3}H]Biotin efflux was measured as previously described.[15] Briefly, PBMC were incubated with [^{3}H]biotin at 475 pmol/L at 37°C for 2 hours to load the cells with [^{3}H]biotin. The cells then were transferred into biotin-free medium that contained pantothenic acid at 1,000 nmol/L; controls were incubated without pantothenic acid in the medium. Incubation was continued at 37°C for 4 hours. In timed intervals, aliquots of the PBMC suspensions were collected and assayed for intracellular [^{3}H]biotin as described above.

Statistics

In the studies of biotin uptake and efflux in the presence of pantothenic acid, significance of differences between groups (e.g., concentration of pantothenic acid) was tested by repeated measures analysis of variance (ANOVA) using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA USA); this procedure takes into account the fact that variability among observations taken from the same subject might be smaller than the variability measured between different subjects.[16] Repeated measures ANOVA is powerful in detecting significant differences among experimental groups in spite of substantial differences among individuals undergoing repeated observations. In the pantothenic acid analogs experiments, significance of differences among groups was tested by one-way ANOVA using SuperANOVA 1.11 (Abacus Concepts, Inc.). Group values were expressed as percent of controls to have a common denominator (100%) for all subjects; values were log transformed before ANOVA analysis to adjust for potentially heterogeneous variances. Significance of differences for cell viability (before vs. after incubation), maximal transport rate (control vs. pantothenic acid), and Michaelis constant (control vs. pantothenic acid) was tested by paired, two-tailed *t*-test. Differences were considered significant if the *P*-value was less than 0.05. Values are expressed as mean ± 1 SD.

Results

Initially we assessed the effect of pantothenic acid on the transport of biotin into PBMC. The concentrations of pantothenic acid spanned a range of 0 to 1,000 nmol/L; [^{3}H]biotin concentrations were either 475 or 1,900 pmol/L. For both vitamins, these concentrations are similar to or moderately greater than the concentrations found in untimed plasma from normal subjects.[6,17] At a [^{3}H]biotin concentration of 475 pmol/L, pantothenic acid slightly decreased biotin uptake compared with controls without pantothenic acid ($P = 0.03$ by repeated measures ANOVA; *Figure 1*). This effect was greatest at 1,000 nmol/L pantothenic acid; at that concentration, biotin uptake into PBMC decreased by only 12% to 88 ± 7% of control values. At a more physiologic concentration of pantothenic acid (100 nmol/L), biotin uptake into PBMC decreased by only 5% to 95 ± 6% of control values. Pantothenic acid reduced the uptake of biotin into PBMC in every experiment in which pantothenic acid concentration was greater than 100 nmol/L. Hence, it seems likely that pantothenic acid has a small but reproducible effect on biotin uptake into PBMC.

Likewise, at 1,900 pmol/L [^{3}H]biotin, a progressive but small decrease of biotin uptake was observed with increasing pantothenic acid ($P = 0.007$; *Figure 1*). The greatest effect (a decrease by 10% to 90 ± 7%) was observed at 1,000 nmol/L pantothenic acid.

We sought to characterize the mechanism(s) by which pantothenic acid caused the small but reproducible inhibition of biotin uptake into PBMC. We incubated PBMC with

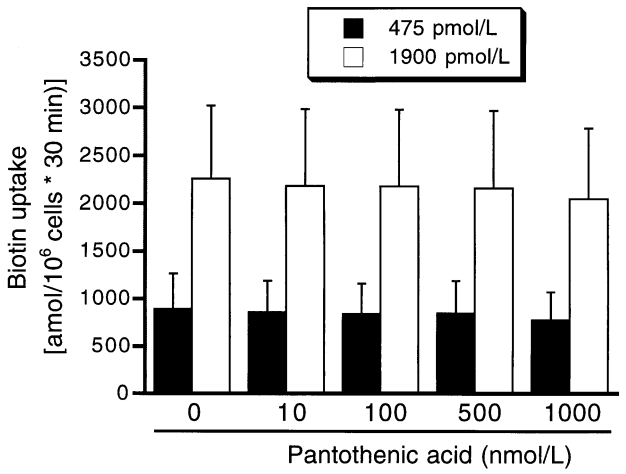

Figure 1 Transport of [^{3}H]biotin into human peripheral blood mononuclear cells at various concentrations of D-pantothenic acid (0 to 1,000 nmol/L medium). Biotin concentrations in the medium were either 475 or 1,900 pmol/L. Values are means ± SD, $n = 5$ subjects ($P = 0.03$ at a biotin concentration of 475 pmol/L; $P = 0.007$ at a biotin concentration of 1,900 pmol/L; statistical analysis by repeated measures analysis of variance).

either 100 nmol/L pantothenic acid or no pantothenic acid (control). [^{3}H]Biotin concentrations were varied from 238 to 2,850 pmol/L. The Lineweaver-Burk plots of biotin uptake in the presence and absence of pantothenic acid intersected near the y-axis (*Figure 2*), suggesting reversible competitive inhibition. The maximal transport rates (V_{max}) for biotin uptake were 3,163 ± 1,379 amol/(10^6 cells × 30 min) and 3,063 ± 1,244 amol/(10^6 cells × 30 min) in the presence and absence of pantothenic acid, respectively, and were not significantly different. The Michaelis constants (K_m) of biotin were 2.0 ± 0.5 nmol/L and 1.6 ± 0.5 nmol/L

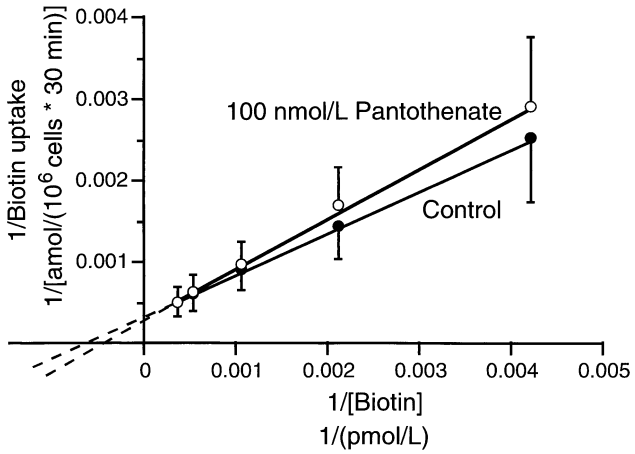

Figure 2 Lineweaver-Burk plot of the [^{3}H]biotin transport into human peripheral blood mononuclear cells in the presence of D-pantothenic acid (100 nmol/L medium); controls were incubated without pantothenic acid. Biotin concentrations in the medium span the range of 238 to 2,850 pmol/L. Regression lines: y = 0.567x ± 0.00036 ($r = 0.994$ ± 0.007), controls; y = 0.668x ± 0.00035 ($r = 0.996$ ± 0.006), 100 nmol/L pantothenic acid. Values are means ± SD, $n = 5$ subjects.

in the presence and absence of pantothenic acid, respectively, ($P < 0.05$).

We examined structural requirements for recognition of pantothenic acid by the biotin transporter in PBMC by incubating PBMC with biotin at a concentration of 475 pmol/L and pantothenic acid or pantothenic acid analogs at concentrations of 1,000 nmol/L; controls contained neither pantothenic acid nor any analog. The following analogs were used (*Figure 3*): D-pantothenic acid, D-pantothenyl alcohol (the carboxyl group of pantothenic acid is replaced by an alcohol group), β-alanine (an integral part of the pantothenic acid molecule), D-pantethine (a disulfide dimer of pantetheine, which is an intermediate in the biosynthesis of coenzyme A), *d*(-)-pantoyllactone (an intermediate in the chemical synthesis of pantothenic acid), and coenzyme A (the biologically active form of pantothenic acid).

Neither pantothenic acid nor any of the pantothenic acid analogs inhibited biotin uptake into PBMC significantly ($P = 0.13$ by one-way ANOVA). This series of experiments confirmed that pantothenic acid decreased biotin uptake by 16% to 84 ± 7% of control values (*Figure 4*); this degree of inhibition is similar to that observed previously (see above). If only the effect of pantothenic acid was compared with control, the difference was significant, which agrees with the data shown in *Figure 1* ($P = 0.005$ by paired, two-tailed *t*-test). No pantothenic acid analog reduced biotin uptake more than pantothenic acid per se; values for transport ranged from 85 ± 15% to 93 ± 7% of control values depending on the analog. The similarity of the effects did not provide evidence for specific structural requirements for the modest but reproducible competition between pantothenic acid and biotin.

Of note, the biotin uptake rate in the absence of pantothenic acid that we observed in *Figure 4* was smaller than the biotin uptake rate observed in *Figure 1* under the same conditions [577 ± 148 vs. 893 ± 370 amol/(10^6 cells × 30 min)]. However, this difference falls easily within the threefold range of interindividual variation observed in these studies.

We examined whether pantothenic acid in the extracellular medium would alter the efflux of intracellular biotin (e.g., by a countertransport effect). Previously, we showed that extracellular biotin and some biotin analogs substantially increase the efflux rate for intracellular [^{3}H]biotin from PBMC.[15] In the present study, we transferred PBMC that had been previously loaded with [^{3}H]biotin into medium that contained pantothenic acid at a concentration of 1000 nmol/L. Pantothenic acid in the medium did not significantly alter biotin efflux from PBMC (*Figure 5*).

Discussion

In PBMC, biotin is cotransported with sodium by an energy-requiring process.[14] The study presented here provides evidence that inhibition of biotin transport into PBMC by pantothenic acid is quantitatively minor. Even at a pantothenic acid concentration (1,000 nmol/L) that exceeded by 10-fold the concentrations normally found in plasma, biotin uptake was reduced by only 12% compared with controls. This change is not likely to be physiologically important.

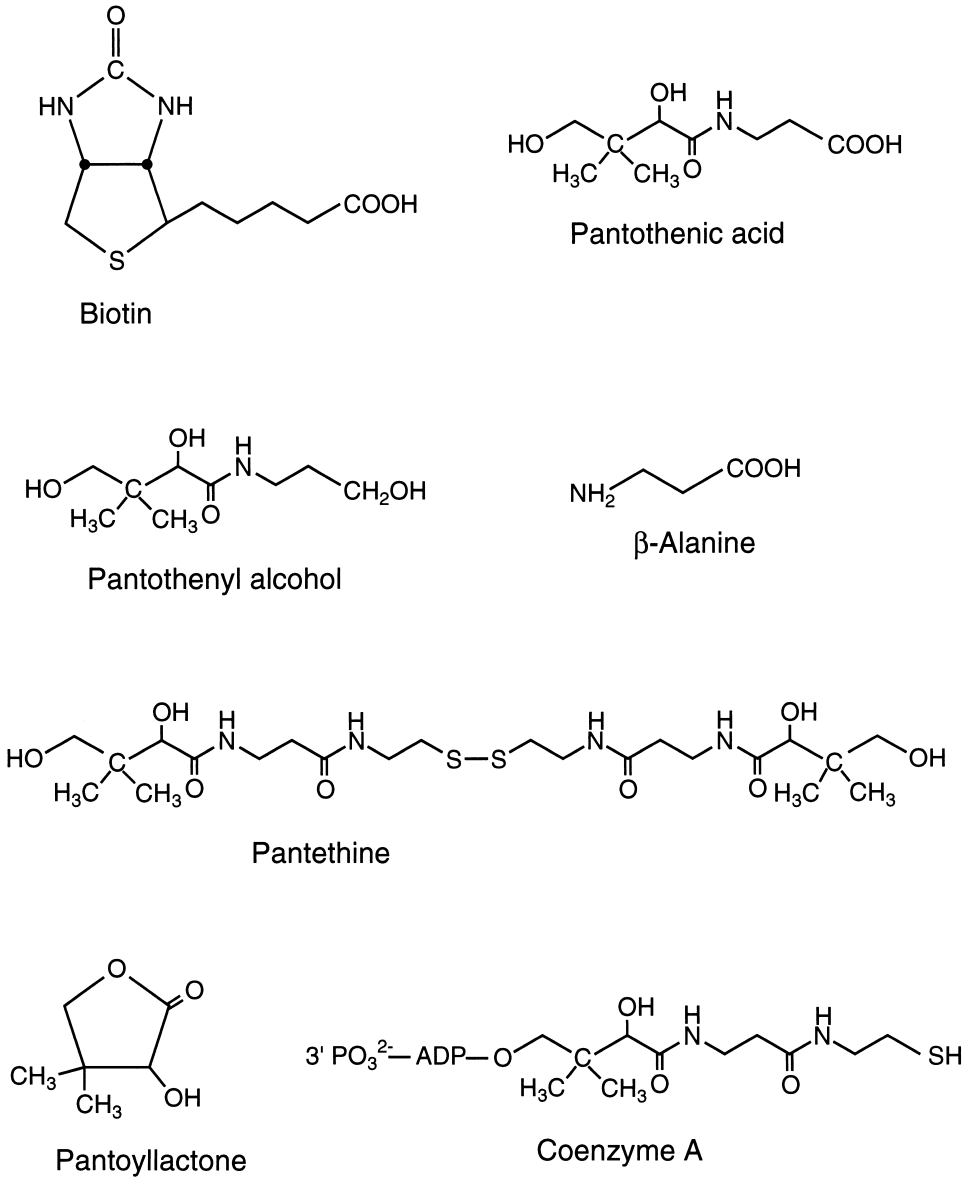

Figure 3 Chemical structures of biotin, pantothenic acid, and pantothenic acid analogs. ADP, adenosine diphosphate.

The minor decrease of biotin uptake into PBMC in the presence of pantothenic acid was caused by reversible competition for binding to the transporter. The regression lines of Lineweaver-Burk plots of biotin transport in the presence or absence of pantothenic acid intersected near the y-axis, suggesting reversible competitive inhibition.[18] This interpretation was supported by statistical analysis of V_{max} and K_m values. V_{max} values were not significantly different between pantothenic acid-containing medium and controls; K_m in pantothenic acid-containing medium was significantly greater than in controls. Nevertheless, inhibition of biotin uptake by pantothenic acid was quantitatively minor.

No dominant structural requirement for inhibition of biotin transport by pantothenic acid was apparent for the biotin transporter in PBMC. Inhibition by pantothenic acid analogs varied from 7 to 16% and thus, the difference between any pair of pantothenic acid analogs was small. It is worth noting that pantothenic acid had the greatest inhibitory effect of all compounds tested and that analogs without a carboxyl group (i.e., pantethine, pantothenyl alcohol) had the smallest effect. We speculate that the carboxyl group might be involved in binding to a region of the transporter that recognizes both biotin and pantothenic acid. This is consistent with previous observations by Bowers-Komro and McCormick[19] in isolated rat hepatocytes; in these studies, a sodium-dependent acid-anion carrier mediates biotin uptake into hepatocytes. Of note, biotin transport into PBMC seems to be more specific than transport into hepatocytes in view of the observation that acids anions such as hexanoic acid, bilirubin, and lipoic acid do not affect biotin transport into PBMC.[14]

Pantothenic acid did not increase biotin efflux from PBMC. In previous studies we showed that extracellular biotin and biotin analogs increase the efflux of intracellular [^{3}H]biotin two times compared with controls.[15] The finding in the present study that pantothenic acid did not increase

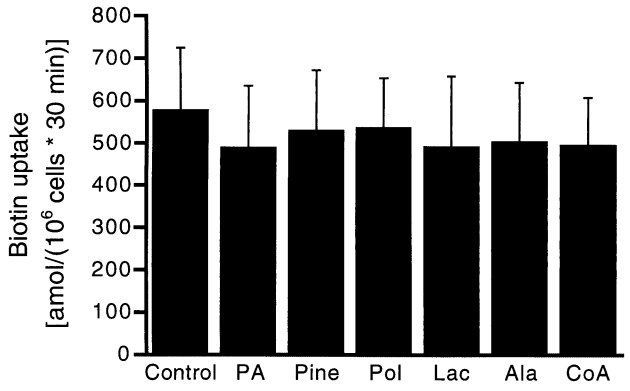

Figure 4 The effects of pantothenic acid and analogs (all at 1,000 nmol/L medium) on the transport of [^{3}H]biotin into human peripheral blood mononuclear cells; controls were incubated without pantothenic acid or analogs. Biotin concentration in the medium was 475 pmol/L. PA, D-pantothenic acid; Pine, D-pantethine; Pol, D-pantothenyl alcohol; Lac, D(-)-pantoyllactone; Ala, β-alanine; CoA, coenzyme A. Values are means ± SD, n = 5 subjects (P = 0.13 among groups as per by one-way analysis of variance).

efflux of intracellular [^{3}H]biotin provides additional though indirect evidence that the transporter responsible for the majority of biotin uptake into PBMC does not recognize pantothenic acid.

The minor effect of pantothenic acid regarding biotin transport in the present study is strikingly different from what has been reported in other cells. For example, Michaelis constants of pantothenic acid and biotin for the cloned and functionally expressed multivitamin transporter from rat placenta were 4.9 and 15.1 μmol/L, respectively[5]; the similar values allow mathematically for a strong interaction. Indeed in this same system, pantothenic acid reduced biotin transport by greater than 90% compared with control values.

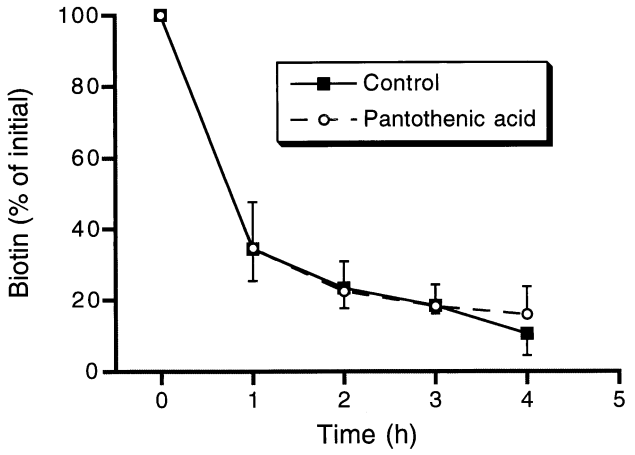

Figure 5 The effects of D-pantothenic acid on the efflux of [^{3}H]biotin from human peripheral blood mononuclear cells (PBMC). PBMC were loaded with [^{3}H]biotin (475 pmol/L medium) at 37°C for 2 hours in pantothenic acid-free medium before they were transferred into biotin-free medium that contained 1,000 nmol/L pantothenic acid. Values are means ± SD, n = 8 to 12 experiments in PBMC from two subjects (P > 0.05, control vs. pantothenic acid, by repeated measures analysis of variance). The coefficient of variation among cells was 14% at time zero.

In the isolated perfused rat brain, pantothenic acid reduced the penetration of biotin through the blood-brain barrier by greater than 80% compared with controls.[4] Finally, in human placental choriocarcinoma cells in culture, biotin reduced the transport of radiolabeled pantothenic acid by greater than 80% compared with control values.[1]

We offer the following explanations for the differences between results of this study and previous studies. First, in the present study we used a different cell line (PBMC) compared with the studies cited above and in the Introduction.[1–3] Second, we have shown that the biotin transporter in PBMC is structurally specific for biotin compounds and does not recognize lipoic acid, hexanoic acid, and bilirubin.[14] This finding is consistent with the observations made in the present study regarding the effects of pantothenic acid. The existence of a transporter that recognizes biotin but not pantothenic acid is important to maintain cellular biotin status in an environment with substantially greater concentrations of pantothenic acid compared with biotin. Nevertheless, a transporter that binds both biotin and pantothenic acid[5] might be present in PBMC and might account for the small but reproducible effect that pantothenic acid had on biotin uptake.

Third, in the previous studies either biotin concentrations, pantothenic acid concentrations, or both were moderately to substantially greater than those normally encountered in human plasma and thus may have led to masking of a transporter of low V_{max} resembling the PBMC transporter. For example, in our studies of biotin transport across the blood-brain barrier and the published studies using the functionally expressed vitamin transporter from rat placenta, biotin concentrations were 3 nmol/L and 35 nmol/L, respectively[4,5]; the biotin concentration in normal human plasma is approximately 0.5 nmol/L.[17] In the same transport studies, pantothenic acid concentrations were 250 μmol/L and 100 μmol/L, respectively[4,5]; the pantothenic acid concentration in normal human plasma is 0.1 μmol/L.[6] In the study that addressed the inhibition of pantothenic acid transport in human placental choriocarcinoma cells by biotin, biotin concentrations were approximately 2×10^5 times higher than the physiologic serum concentrations.[1]

In other cell lines, the K_m values of transporters for biotin was in the micromolar range.[5,20] In the present study, the concentrations of biotin and pantothenic acid in the medium were in the nanomolar range. Thus, one may ask whether the concentrations of biotin and pantothenic acid in this study have been chosen appropriately. Theoretically, if both [biotin] and [pantothenic acid] were $<< K_m$ for biotin, unoccupied carriers could have transported biotin despite pantothenic acid binding to the transporter. Our data suggest that this was not the case in this study. The K_m value of the transporter was 1.6 nmol/L, confirming our previous studies.[14] The biotin concentrations in the medium were 0.5 or 1.9 nmol/L; the pantothenic acid concentrations in most experiments was 100 or 1,000 nmol/L. Thus, the biotin transporter would have been saturated if pantothenic acid had bound even 1/100th as tightly to the transporter as biotin ([pantothenic acid] $> K_m$ for biotin).

In summary, we conclude that PBMC accumulate biotin by a transporter that is specific for biotin. The present study and previous studies provide evidence that another, less

specific transporter in mammalian cells may bind biotin, pantothenic acid, and similar compounds. However, the contribution of this transporter to biotin uptake into PBMC is quantitatively minor. We speculate that pantothenic acid is unlikely to confound studies of biotin nutritional status using biotin concentrations or carboxylase activities in PBMC as indices. In addition, pantothenic acid in incubation medium is unlikely to confound transport studies of biotin in PBMC.

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