Proliferation of Peripheral Blood Mononuclear Cells Increases Riboflavin Influx (44554)

JANOS ZEMPLENI AND DONALD M. MOCK[1]

Department of Pediatrics, University of Arkansas for Medical Sciences and the Arkansas Children's Hospital Research Institute, Little Rock, Arkansas 72202

Abstract. **Previously we demonstrated that proliferation of peripheral blood mononuclear cells (PBMC) causes a five-fold increase in cellular uptake of biotin; this increase is mediated by an increased number of biotin transporters on the PBMC surface. In the present study, we investigated the specificity of this phenomenon by determining whether the cellular uptake of riboflavin also increases in proliferating PBMC and whether the increase is also mediated by an increased number of transporters per cell. We characterized [^{3}H]riboflavin uptake in both quiescent and proliferating PBMC. In quiescent PBMC, [^{3}H]riboflavin uptake exhibited saturation kinetics and was reduced by addition of unlabeled riboflavin ($P < 0.05$) or lumichrome ($P < 0.01$). These observations are consistent with transporter-mediated uptake. [^{3}H]Riboflavin uptake was reduced at 4°C compared with 37°C ($P < 0.01$) and by 2,4-dinitrophenol ($P < 0.05$) but not by ouabain or incubation in sodium-free medium. These data provide evidence for an energy-dependent but sodium-independent transporter. Proliferating PBMC accumulated approximately four times more [^{3}H]riboflavin than quiescent PBMC ($P < 0.05$). Because both transporter affinity and transporter number per cell (as judged by maximal transport rate) were similar in quiescent and proliferating PBMC, we hypothesize that the increased riboflavin uptake by proliferating PBMC reflects only increased cellular volume. To test this hypothesis, PBMC volume was reduced using hyperosmolar medium; [^{3}H]riboflavin uptake decreased to about 50% of isotonic controls ($P < 0.01$). Thus we conclude that proliferating PBMC increase cellular content of riboflavin and biotin by two different mechanisms.**

[P.S.E.B.M. 2000, Vol 225:72–79]

Peripheral blood mononuclear cells (PBMC) represent a heterogeneous population of immune cells (B cells, T cells, and various granulocytes) that arise from pluripotent hematopoietic stem cells in the bone marrow (1). PBMC account for cellular and humoral immune responses; some PBMC (B and T cells) have the inherent ability to proliferate rapidly after antigenic and mitogenic stimulation. PBMC often have been used in models of nutrition research; for example, vitamin status can be assessed by measuring activities of vitamin-dependent enzymes in PBMC (2,3), and vitamin uptake has been studied in PBMC (4–6).

We have recently shown that proliferation of PBMC has substantial effects on cellular biotin metabolism. For example, proliferating PBMC increase biotin uptake five fold compared to quiescent PBMC; this increase is mediated by an increased number of biotin transporters on the cell surface (7). Moreover, proliferating PBMC use biotin to increase activities of biotin-dependent carboxylases (8) and increase biotinylation of histones (9). These observations raise the following questions about the effect of proliferation on the cellular status of other vitamins in PBMC: (i) Do vitamin status assessments that are based on cellular vitamin concentrations need to be adjusted for the physiologic state of the cells (e.g., proliferating versus nonproliferating cells)? (ii) Does the vitamin requirement of cells increase

This research was supported by NIH grant DK 36823 (D.M.M.) and CUMG grant 117-340-3075 (J.Z.).

[1] To whom requests for reprints should be addressed at the Arkansas Children's Hospital, Pediatric Gastroenterology, mail slot 512, 800 Marshall Street, Little Rock, AR 72202. E-mail: mockdonaldm@exchange.uams.edu

Received January 31, 2000. [P.S.E.B.M. 2000, Vol 225]
Accepted April 28, 2000.

0037-9727/00/2251-0072$15.00/0

with proliferation? (iii) Does proliferation of cells (e.g., immune cells or rapidly growing fetal tissues) cause an increased vitamin requirement of the whole organism? (iv) Do vitamins such as biotin play important roles in cell cycling and gene expression, perhaps acting by covalent modification of histones by biotin?

The overall goal of the studies presented here was to determine whether proliferation of PBMC affects a second water-soluble vitamin, riboflavin. We chose riboflavin because riboflavin uptake into cells is mediated by a distinct transporter that shares few characteristics with the biotin transporter. For example, previous studies in various cell lines concluded that cellular uptake of riboflavin is not sodium dependent (10–14) whereas the biotin uptake into PBMC is sodium dependent (4). Also, riboflavin does not compete with biotin for uptake into renal epithelial cells or liver cells (13, 14). Moreover, riboflavin and biotin are not structurally similar and are metabolized by very different pathways (15–19).

Specifically we sought to (i) identify the mechanism(s) for uptake of riboflavin in PBMC; (ii) determine whether proliferation causes an increased riboflavin uptake into PBMC; and (iii), if increased, determine whether the increased riboflavin uptake is mediated by an increased number of transporters per cell, as has been observed for biotin.

Materials and Methods

Materials. [^{3}H]Riboflavin (specific radioactivity 2.15 TBq/mmol) was purchased from Sigma (St. Louis, MO). Radiochemical purity of [^{3}H]riboflavin was greater than 95% as determined by thin-layer chromatography (see below). To avoid photodecomposition, riboflavin and its analogs were protected against exposure to light by using amber vials and aluminum foil. [^{3}H]Thymidine (specific radioactivity 1.29 TBq/mmol) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Riboflavin-free RPMI-1640 was prepared by Atlanta Biologicals (Norcross, GA). All other chemicals were purchased from Sigma.

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

Fifteen subjects (10 women) donated blood for isolation of PBMC; most subjects donated blood on more than one occasion (i.e., for different experiments). The age of the subjects spanned a range of 28–52 years; all subjects were nonsmokers. None of the subjects consumed vitamin supplements that contained more than the Recommended Dietary Allowance of riboflavin (20). Individuals receiving thyroid hormone therapy were excluded because thyroid hormones interfere with riboflavin metabolism (21, 22).

Isolation of PBMC. PBMC were isolated from an individual's blood by gradient centrifugation as described previously (4, 23). The studies described below were performed using either quiescent, nonproliferating PBMC or mitogen-stimulated, proliferating PBMC.

Quantitation of [^{3}H]Riboflavin Uptake and Metabolism in Quiescent PBMC. The PBMC pellet from 120 ml of blood was suspended in ≈ 30 ml of phosphate-buffered saline containing 5.5 m*M* D-glucose. Cell density in the suspension and cell viability were determined as described before (7). Cell density was $5.3 \pm 1.6 \times 10^6$ cells/ml; viability was greater than 99%. The uptake rates of [^{3}H]riboflavin were normalized by 10^6 viable cells.

Cellular riboflavin uptake was measured in 1-ml aliquots of the PBMC suspension, as described previously, with one alteration (4); [^{3}H]biotin was replaced with [^{3}H]riboflavin. Transport was studied in the linear range, and effects of inhibitors and flavin analogs were studied as described previously (4). Typically, [^{3}H]riboflavin uptake over a 10-min period was determined at 37°C using a physiologic concentration of riboflavin (10 n*M*) in the medium (24). Immediately after incubation, extracellular [^{3}H]riboflavin was removed by washing with phosphate-buffered saline followed by liquid scintillation counting of the cell pellet as described before (4). We confirmed that PBMC do not release measurable quantities of intracellular [^{3}H]riboflavin during washing with phosphate-buffered saline at 4°C (data not shown). Blanks were prepared by replacing PBMC suspension with cell-free medium; blank values were subtracted from samples values.

For solubility reasons, some transport inhibitors and flavins had to be dissolved in either dimethyl sulfoxide (riboflavin, ouabain, 2,4-dinitrophenol) or methanol (lumichrome). To exclude solvent artifacts, we assessed [^{3}H]riboflavin uptake in the presence of dimethyl sulfoxide and methanol at the highest final concentration of these solvents used in the current studies (5% by vol); neither significantly affected riboflavin uptake (data not shown).

To investigate whether PBMC metabolize riboflavin during transport studies, we incubated the cells with 10 n*M* [^{3}H]riboflavin at 37°C. At timed intervals (10, 30, 90 min after addition of [^{3}H]riboflavin), PBMC were harvested by centrifugation at 2260*g* for 90 sec; PBMC pellets were suspended in 40 μl of phosphate-buffered saline, followed by lysis with 5 μl 0.5% Triton X-100 and 5 μl of 2 *M* trichloroacetic acid. After vortexing, 10 μl of this lysate were spotted onto a silica gel plate for thin-layer chromatography; the plate was developed in the dark using benzene: 1-butanol: methanol: water (1: 2: 1: 1, by vol; upper phase) as solvent (16). Lanes of the dried chromatography plate were cut into equal pieces, and radioactivity was determined by liquid scintillation counting of the pieces. Authentic standards of riboflavin, flavin adenine dinucleotide, flavin mononucleotide, and lumichrome were run on the same plate and located under a Mineralight lamp (16).

Quantitation of [^{3}H]Riboflavin and [^{3}H]Thymidine Uptake in Mitogen-Stimulated, Proliferating PBMC. PBMC were isolated aseptically as described before (7). After the final wash, the PBMC pellet was suspended in sterile-filtered RPMI-1640 (originally free of riboflavin) containing 10% autologous plasma (v/v), 100

I.U./ml penicillin, and 100 μg/ml streptomycin; unlabeled riboflavin was added to produce a final concentration of 5 n*M*. The cell number per ml of medium was adjusted to 4 × 10^6 PBMC/ml by dilution with medium. Then, either the T-cell mitogen concanavalin A (final concentration 20 μg/ml) or the T and B cell mitogen pokeweed lectin (2.0 μg/ml; from *Phytolacca americana*) was added to the PBMC suspension. These suspensions were then incubated at 37°C for 3 days in an atmosphere of 95% air and 5% CO_2 to induce proliferation; nonproliferating controls were incubated without mitogen. After incubation, cell numbers per ml and cell viability were determined again (7). The uptake rates of [^{3}H]riboflavin were determined as described above; the specific radioactivity of [^{3}H]riboflavin was adjusted for the concentration of unlabeled riboflavin in the medium. The uptake rates of [^{3}H]thymidine were determined as described previously (7).

Statistics. Significance of differences among groups (e.g., among the two different mitogens and the control) were tested by one-way ANOVA. Dunnett's *posthoc* procedure was used for *posthoc* testing; Dunnett's compares the mean of each treatment group (e.g., mitogen-stimulated cells) to that of a designated control (e.g., unstimulated cells) (25, 26). Effects of incubation time and temperature were tested by two-way ANOVA (time by temperature). Paired comparisons were made using the paired, two-tailed *t* test. SuperANOVA 1.11 and StatView 4.5 (Abacus Concepts, Berkeley, CA) were used to perform all calculations. Differences were considered significant if $P < 0.05$. Popular central tendency and dispersion are expressed as mean ± 1 SD.

Results

We initially determined whether uptake of [^{3}H]riboflavin into PBMC was temperature dependent and whether uptake increased linearly with time. Riboflavin uptake into quiescent PBMC was significantly greater at 37°C than at 4°C (Fig. 1). When quiescent PBMC were incubated with physiologic concentrations of riboflavin (10 n*M*), the mean riboflavin uptake of five subjects increased linearly with incubation time for at least 20 min (Fig. 1). Because riboflavin uptake into PBMC of two of these subjects exhibited a tendency to plateau after about 15 min, an incubation time of 10 min was used for all subsequent experiments. Riboflavin uptake into PBMC also increased linearly with time (r = 0.949) when PBMC were incubated with a pharmacologic concentration of riboflavin (i.e., 1000 n*M*). Thus, the studies of saturation kinetics (see below) were not confounded by saturating transport prematurely (i.e., before termination of the 10-min incubations) when pharmacologic concentrations of [^{3}H]riboflavin were used.

To assess whether riboflavin uptake into quiescent PBMC exhibits saturation kinetics, PBMC were incubated with [^{3}H]riboflavin at concentrations between 5 and 1000 n*M*. Using a physiologic concentration of [^{3}H]riboflavin (10 n*M*) in the medium, cellular uptake was 484 ± 212 amol/(10^6 cells × 10 min) at 37°C. In each of the five individuals studied, riboflavin uptake into PBMC approached a plateau at high riboflavin concentrations consistent with saturation kinetics (Fig. 2). Each data set was accurately fit (r = 0.999 ± 0.001) by nonlinear regression using the Michaelis-Menten equation (plots not shown). Mean Michaelis-Menten constant (K_m) was 955 ± 344 n*M*; mean maximal transport rate (V_{max}) was 36.5 ± 20.1 fmol/(10^6 cells × 10 min). These observations concerning temperature and saturation are consistent with carrier-mediated uptake.

We further investigated the putative riboflavin transporter by assessing inhibition of transport by four sugar alcohols (ribitol, xylitol, glucitol, and mannitol) that have structural similarities to the ribityl side chain of riboflavin. We incubated quiescent PBMC with 10 n*M* [^{3}H]riboflavin and 10 μ*M* sugar alcohols (= 1000-fold higher). Uptake of [^{3}H]riboflavin did not decrease significantly compared with control without sugar alcohol (data not shown). To assess the effect of a water-soluble vitamin, similar competition experiments were performed with 10 μ*M* biotin; no significant competition was observed (data not shown).

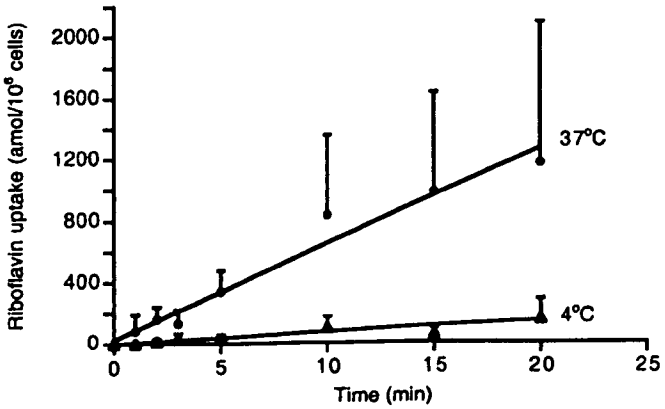

Figure 1. Effects of incubation time and temperature on the uptake of [^{3}H]riboflavin (10 n*M*) into human PBMC. Means ± SD from five subjects are depicted. Effects of time and temperature were both significant ($P < 0.01$ by two-way ANOVA). At 4°C: uptake = 6.7 × time − 1.1 (r = 0.897); at 37°C: uptake = 62 × time + 28 (r = 0.983).

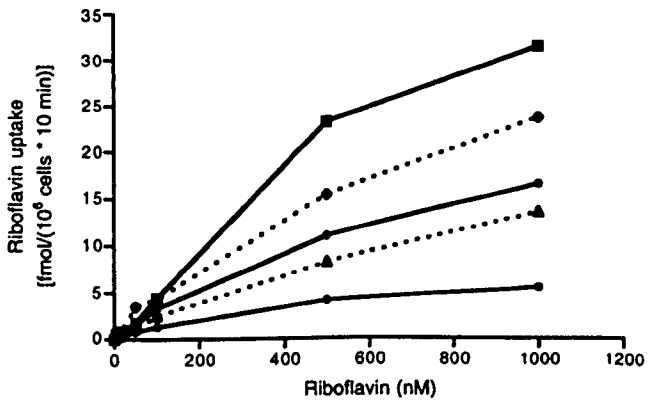

Figure 2. Saturation kinetics of [^{3}H]riboflavin uptake into human PBMC. Data from five subjects are depicted. PBMC were incubated with 5–1000 n*M* [^{3}H]riboflavin at 37°C for 10 min.

To assess the importance of various regions of the riboflavin molecule for binding to the transporter, we conducted a series of competition experiments with riboflavin analogs of 1000-fold greater concentration. Quiescent PBMC were incubated with 10 n*M* [^{3}H]riboflavin in the presence (10 μ*M*) of either unlabeled riboflavin (intact isoalloxazine ring and ribityl side chain), flavin mononucleotide (FMN; ribityl side chain is phosphorylated), ribose (analog of the ribityl side chain), lumichrome (intact isoalloxazine ring without ribityl side chain), or flavin adenine dinucleotide (adenosine diphosphate attached to the ribityl side chain); the control was incubated without unlabeled riboflavin or any analog. The uptake of [^{3}H]riboflavin was reduced significantly by unlabeled riboflavin and lumichrome to 53 ± 22% and 38 ± 13%, respectively, of control (Fig. 3). FMN reduced the cellular uptake of [^{3}H]riboflavin to 64% of control values; however, this effect did not reach statistical significance. Analysis by thin-layer chromatography revealed that FMN did not contain detectable amounts of riboflavin impurities. Further, PBMC did not hydrolyze FMN in the extracellular medium (data not shown). Thus, the partial effect of FMN on cellular [^{3}H]riboflavin uptake is attributable to FMN rather than riboflavin impurities or FMN hydrolysis. Ribose and flavin adenine dinucleotide did not significantly affect the cellular uptake of [^{3}H]riboflavin. These findings suggest that the ribityl side chain is not important for binding to the transporter because ribose (side chain analog) did not compete for cellular uptake, whereas lumichrome (missing side chain) did compete for cellular uptake. However, the data indicate that increasing the size of the molecule, either by adding phosphate or adenosine diphosphate to the side chain can reduce the ability to bind to the transporter, suggesting a steric effect. Overall, these data provide evidence that a transporter mediates at least some of the cellular uptake of riboflavin.

We further characterized the transporter as follows: PBMC were incubated with [^{3}H]riboflavin in the presence of one of the following transport inhibitors (all at 1 m*M*): ouabain (an inhibitor of Na-K-ATPase), iodoacetate (a compound that covalently modifies sulfhydryl groups), or 2,4-dinitrophenol (a respiratory chain inhibitor that reduces intracellular ATP levels); controls were incubated without inhibitors. Ouabain did not significantly affect riboflavin uptake (Fig. 4), suggesting that riboflavin uptake is not sodium dependent. Iodoacetate did not significantly affect riboflavin uptake. In separate experiments, treatment of PBMC with a different sulfhydryl-modifying agent (0.5 m*M* *p*-hydroxymercuriphenyl sulfonic acid) also caused no significant decrease of riboflavin uptake (data not shown). Taken together, the observations using these two sulfhydryl reagents provide evidence that sulfhydryl groups of the transport protein are not directly involved in uptake. In contrast, treatment with 2,4-dinitrophenol decreased riboflavin uptake to 76 ± 14% of control values ($P < 0.05$), providing evidence that riboflavin uptake is energy dependent.

Absence of cation requirement was further examined in experiments in which PBMC were incubated with [^{3}H]riboflavin in medium containing 150 m*M* choline, lithium, or ammonium rather than sodium; controls were incubated in sodium-containing medium. Replacement of sodium did not significantly affect riboflavin uptake, providing additional evidence that cellular riboflavin uptake does not require co-transport with sodium (data not shown).

Next, we sought to determine whether quiescent PBMC metabolize riboflavin during incubation times used in the transport studies. We incubated PBMC with 10 n*M* [^{3}H]riboflavin and collected cell pellets after 10, 30, and 90 min. Tritium within PBMC was separated by thin-layer chromatography followed by liquid scintillation counting (see Materials and Methods). By 90 min, no metabolism of riboflavin was detectable. Figure 5 depicts the 10-min and 90-min samples. The arrows in Figure 5 denote the chromatographic position expected of flavin metabolites. Riboflavin

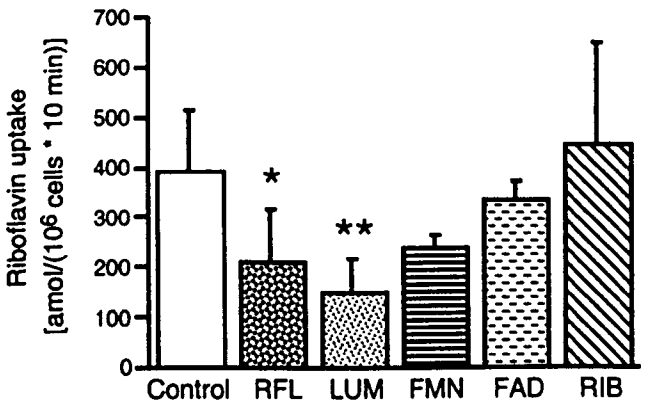

Figure 3. The effects of riboflavin (RFL), lumichrome (LUM), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and ribose (RIB; all at 10 μ*M*) on the uptake of [^{3}H]riboflavin (10 n*M*) into human PBMC. Means ± SD from five subjects are depicted. Controls were incubated without transport competitors. $^*P < 0.05$ versus control; $^{**}P < 0.01$ versus control (by one-way ANOVA and Dunnett's *posthoc* test).

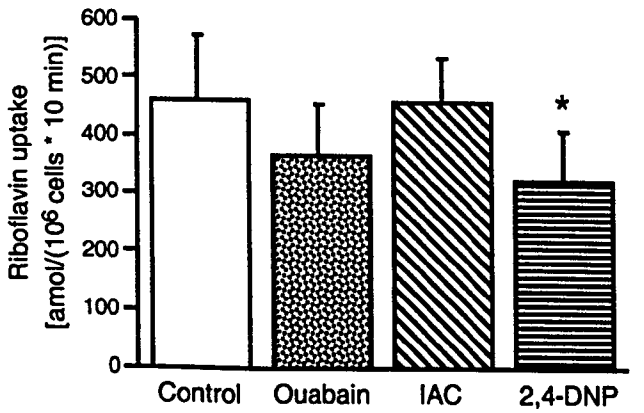

Figure 4. The effects of ouabain, iodoacetate, and 2,4-dinitrophenol (all at 1 m*M*) on the uptake of [^{3}H]riboflavin (10 n*M*) into human PBMC. Means ± SD from seven subjects are depicted. Controls were incubated without transport inhibitors. $^*P < 0.05$ versus control (by one-way ANOVA and Dunnett's *posthoc* test). IAC = iodoacetate; 2,4-DNP = 2,4-dinitrophenol.

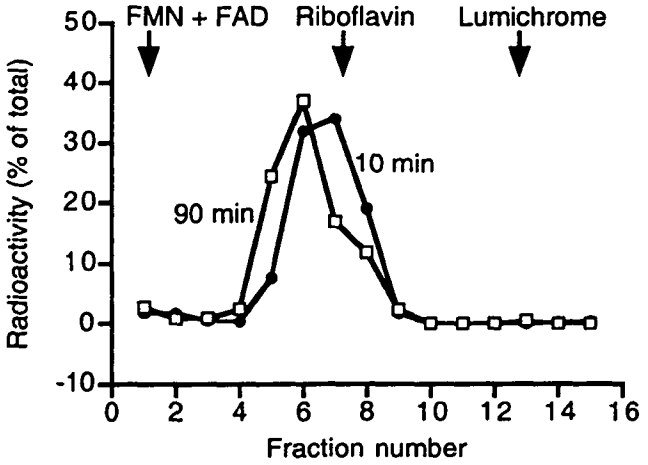

Figure 5. Riboflavin metabolism in quiescent human PBMC. Cells were incubated with [^{3}H]riboflavin (10 n*M*) for 10 or 90 min. Contents of lyzed cells were chromatographed using thin-layer chromatography. The TLC lanes were cut into 15 equal pieces, and flavins were quantitated by liquid scintillation counting of the TLC pieces. Arrows indicate the chromatographic mobility of authentic standards of expected metabolites. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

migrates discretely from the other flavins in this TLC system. In a controlled experiment, we confirmed that sample preparation does not cause acid hydrolysis of flavin adenine dinucleotide.

The effect of PBMC proliferation on the cellular uptake of riboflavin was assessed at a physiologic concentration of [^{3}H]riboflavin. Proliferating PBMC did accumulate significantly more vitamin than unstimulated controls: 261 ± 180 amol/(10^6 cells × 10 min) in quiescent controls; 1088 ± 468 amol/(10^6 cells × 10 min) in concanavalin A–stimulated PBMC; 1015 ± 504 amol/(10^6 cells × 10 min) in pokeweed lectin–stimulated PBMC ($P < 0.05$ by one-way ANOVA and Dunnett's *posthoc* test). Proliferation of mitogen-stimulated PBMC was confirmed by cellular [^{3}H]thymidine uptake. Mitogen-stimulated PBMC accumulated greater than 16 times more thymidine than quiescent controls ($P < 0.01$; data not shown).

To explore the mechanism of increased uptake, quiescent and mitogen-stimulated PBMC were incubated with [^{3}H]riboflavin at concentrations from 5 to 1000 n*M*. As judged by the Michaelis-Menten constants (Table I), proliferation did not affect affinity of the transporter for riboflavin, and the number of riboflavin transporters on the PBMC surface did not change significantly.

By what mechanism could proliferating PBMC increase cellular riboflavin uptake without altering transporter affinity or transporter number per cell? One potential mechanism is increased cellular volume. Such an increase during PBMC proliferation has been reported (27, 28) and could cause increased passive influx (simple diffusion or facilitated diffusion) of riboflavin per cell, thereby increasing the content per cell without increasing the cellular concentration of riboflavin. To assess the effect of altered cell volume, crystalline mannitol was added to the incubation

Table I. Transport Kinetics of Riboflavin in Mitogen-Stimulated PBMC

Treatment	K_m	V_{max}
	n*M*	fmol/(10^6 cells × 10 min)
Concanavalin A	594 ± 395	39 ± 20
Pokeweed lectin	513 ± 470	23 ± 10
Control (no mitogen)	537 ± 375	28 ± 19
P value	NS	NS

Note. PBMC were incubated with either concanavalin A (20 μg/ml) or pokeweed lectin (2.0 μg/ml) to stimulate proliferation; controls were incubated without mitogen. On Day 3, cells were harvested and cellular [^{3}H]riboflavin uptake was measured using [^{3}H]riboflavin concentrations of 5–1,000 n*M* in the medium. K_m (Michaelis-Menten constant) and V_{max} (maximal transport rate) were determined by nonlinear regression. NS = values within the same column are not significantly different by one-way ANOVA ($n = 5$).

medium to produce final osmolarities of either 305 (control), 405, 505, 605, or 805 mOsm; increased osmolarity of the medium causes cell shrinkage (29). PBMC were allowed to equilibrate for at least 30 min at 37°C before transport studies were conducted with [^{3}H]riboflavin. In both quiescent and mitogen-stimulated PBMC, [^{3}H]riboflavin uptake decreased significantly with increasing osmolarity of the medium (Fig. 6). At 805 mOsm, [^{3}H]riboflavin was 38% to 55% of that in isotonic medium. These two to three times reductions are consistent with the hypothesis that most of the increased uptake of riboflavin into proliferating PBMC can be attributed to the increased cellular volume routinely associated with blastogenesis.

Is the increased influx of riboflavin into proliferating PBMC mediated by a transporter or by simple diffusion across the cell membrane? To distinguish between these two mechanisms, we determined uptake of [^{3}H]riboflavin (10 n*M*) in pokeweed-stimulated PBMC in the presence (10 μ*M*) of either unlabeled riboflavin or lumichrome. Both unlabeled riboflavin and lumichrome caused a significant decrease of cellular uptake of [^{3}H]riboflavin (58% ± 70% and 34% ± 13% of control; $P < 0.01$). These findings are consistent with the hypothesis that the increased uptake of riboflavin into proliferating PBMC is a transporter-mediated process.

We also assessed whether proliferating PBMC metabolize riboflavin and thereby enhance net transport by metabolic trapping. We induced proliferation of PBMC by incubation with pokeweed lectin (2.0 μg/ml) for 3 days. The proliferating PBMC were incubated with 10 n*M* [^{3}H]riboflavin for 10, 30, and 90 min. Analysis of lyzed cell pellets by thin-layer chromatography revealed no detectable metabolism (<5%) of riboflavin in proliferating PBMC.

To further assess whether the increased riboflavin uptake in mitogen-stimulated PBMC results from PBMC proliferation, we investigated the time course of stimulation of riboflavin uptake into proliferating PBMC. In these experiments, we added either concanavalin A (final concentration 20 μg/ml) or pokeweed lectin (final concentration 2.0 μg/ml) to a suspension of PBMC (4×10^6 cells/ml). Stimulation

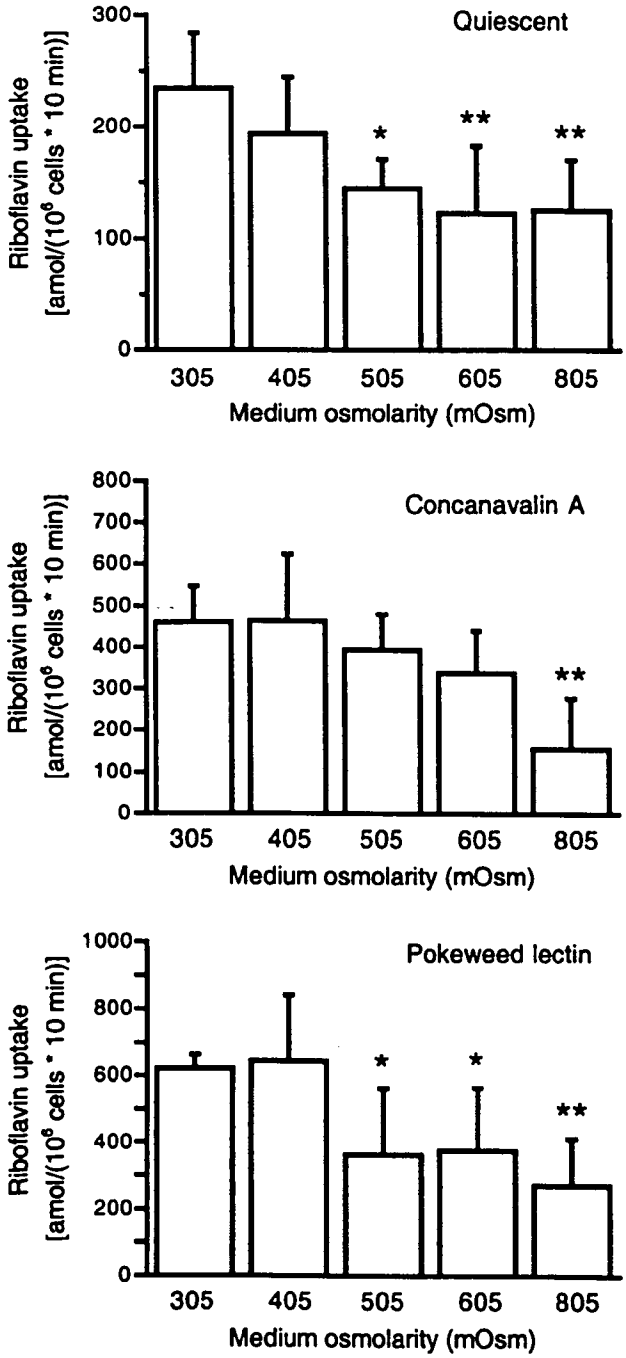

Figure 6. Effects of osmolarity of the medium on riboflavin uptake into human PBMC. Quiescent or mitogen-stimulated PBMC (3 days with either 20 µg/ml concanavalin A or 2.0 µg/ml pokeweed lectin) were transferred into media in which the osmolarities were increased as shown by addition of mannitol; controls were incubated in isotonic (305 mOsm) medium. After 30 min, uptake of 10 n*M* [^{3}H]riboflavin was measured. Means ± SD from three to six samples are depicted. Upper panel, quiescent PBMC; middle panel, concanavalin A-stimulated PBMC; bottom panel, pokeweed lectin-stimulated PBMC. *$P < 0.05$ versus control; **$P < 0.01$ versus control (by one-way ANOVA and Dunnett's *posthoc* test).

of riboflavin uptake reached maximal values 48 hr after addition of either mitogen to the medium (Fig. 7). The increase of riboflavin uptake paralleled the increase of [^{3}H]thymidine uptake.

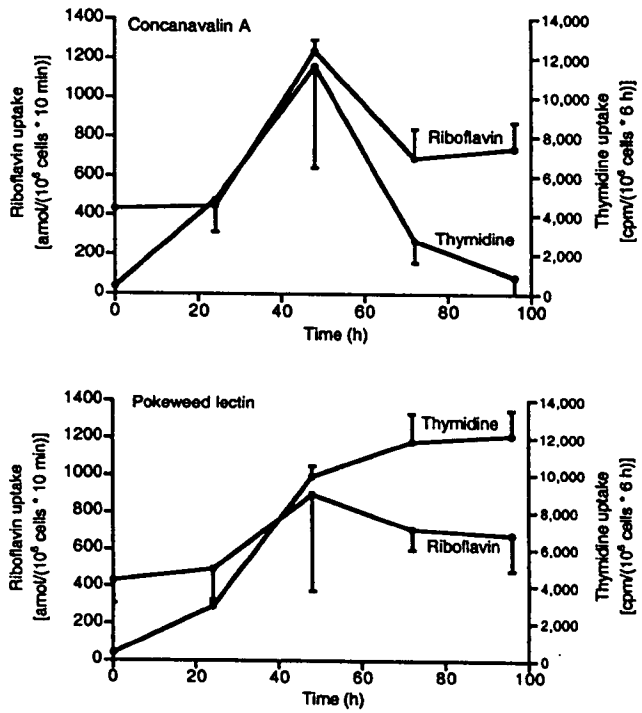

Figure 7. Time course of [^{3}H]riboflavin and [^{3}H]thymidine uptake in mitogen-stimulated human PBMC. Cells were incubated with either 20 µg/ml concanavalin A (upper panel) or 2.0 µg/ml pokeweed lectin (lower panel) for up to 96 hr. At timed intervals, aliquots were collected, and cellular uptake of [^{3}H]riboflavin and [^{3}H]thymidine was measured. Zero-time values were measured before addition of mitogen to the medium. Means ± SD from five to six samples are depicted.

Discussion

The data of this study provide evidence that riboflavin uptake into human PBMC is mediated by a structurally specific, energy-dependent transporter that is not sodium dependent. This conclusion is based on the following observations: (i) riboflavin uptake is temperature dependent; (ii) riboflavin uptake exhibits saturation; (iii) an excess of unlabeled riboflavin and some riboflavin analogs competitively decreases the uptake of [^{3}H]riboflavin; (iv) 2,4-dinitrophenol reduces the uptake of riboflavin; (v) sugar alcohols and biotin do not compete with riboflavin for cellular uptake; (vi) riboflavin uptake does not decrease when extracellular sodium is replaced by other cations; and (vii) ouabain does not affect riboflavin uptake. However, our studies also provide evidence that riboflavin uptake into PBMC is affected by cell volume and thus by diffusion processes (see below).

For riboflavin, the increase is apparently mediated largely by an increase in cell volume during blastogenesis. The mean cell volume of mitogen-stimulated PBMC is two to four times greater than quiescent PBMC (27, 28). There is precedence for such an effect. For nicotinamide dinucleotide (NAD), increased cell volume accounts for the increase of NAD content of PBMC (28). The intracellular NAD concentration remains constant during proliferation whereas the NAD content per cell increases in parallel with the in-

crease in cellular volume. We hypothesize that a similar mechanism accounts for the increased riboflavin content of proliferating PBMC. Observations presented here and reports of others support this hypothesis: (i) If the osmolarity of culture medium is increased by ≈ 76% by increasing from 0.85 to 1.5 g/100 ml sodium chloride, the volume of human PBMC decreases to 70% of isotonic controls (29). (ii) In the present study, when osmolarity of the medium was increased by 67% (from 305 mOsm to 505 mOsm), the riboflavin content of quiescent PBMC decreased to 58% of controls; values for mitogen-stimulated PBMC were similar. Taken together, these studies suggest that net riboflavin uptake into PBMC is directly affected by cellular volume.

The volume-augmented increase of riboflavin uptake is likely to have been augmented by facilitated diffusion. For example, rat liver cells accumulate riboflavin by facilitated diffusion followed by metabolic trapping by flavokinase-catalyzed phosphorylation to flavin mononucleotide; K_m for this transporter is ≈ 12 μM (10). Unless such metabolic trapping occurs, diffusion processes cannot generate concentration gradients across cell membranes; the concentration inside the cell will approach the concentration outside the cell. We propose that diffusion without metabolic trapping likely accounts for the increased riboflavin uptake into proliferating PBMC for the following reasons: (i) Michaelis-Menten constant and maximal transport rates were similar in quiescent and proliferating PBMC, suggesting that substrate affinity and transporter number per cell did not change during proliferation. (ii) In both quiescent and proliferating PBMC, cellular uptake of [^{3}H]riboflavin was inhibited by excess of unlabeled riboflavin or lumichrome suggesting that [^{3}H]riboflavin uptake is mediated by facilitated diffusion rather than by simple diffusion across the cell membrane. (iii) Reduction of cellular volume in hyperosmolaric medium decreased riboflavin uptake. (iv) PBMC did not use [^{3}H]riboflavin to synthesize flavin mononucleotide or flavin adenine dinucleotide during our short-term incubations for up to 90 min, suggesting that metabolic trapping does not occur in PBMC. However, we cannot exclude the possibility that binding of riboflavin to intracellular immunoglobulins in secretory vesicles may transiently augment the influx of riboflavin. Immunoglobulins are quantitatively important riboflavin-binding proteins in plasma (30). Whether the increased riboflavin content but constant concentration of proliferating PBMC is a response to increased cellular demand is unclear from the current study.

Most likely, the riboflavin transporter in human PBMC is similar to the riboflavin transporters in *Xenopus laevis* oocytes, HK-2 cells (a human renal proximal tubule epithelial cell line), rat liver basolateral membrane vesicles, and human-derived liver cells HepG2 (11–14). This conclusion is based on the observation that the affinities for riboflavin are very similar; K_m values range from 410 to 3550 nM. Substrate specificities are also similar, and riboflavin uptake is sodium independent for all these transporters.

In summary, the studies presented here provide evidence that uptake of riboflavin and biotin occurs by different mechanisms. Increased riboflavin uptake into proliferating PBMC appears to result primarily from an increase in cell volume. In contrast, the increase of biotin uptake is likely mediated by transporter synthesis. We speculate that the increase of biotin uptake may be a response to increased demand for biotin-dependent carboxylases (8) or biotinylation of histones (9) or both.

1. Janeway CA, Travers P, Walport M, Capra JD. Immuno Biology (4th ed). London: Garland Publishing/Elsevier, 1999.
2. 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 **44**:11–16, 1990.
3. Velazquez A, Teran M, Baez A, Gutierrez J, Rodriguez R. Biotin supplementation affects lymphocyte carboxylases and plasma biotin in severe protein-energy malnutrition. Am J Clin Nutr **61**:385–391, 1995.
4. Zempleni J, Mock DM. Uptake and metabolism of biotin by human peripheral blood mononuclear cells. Am J Physiol **275**:C382–C388, 1998.
5. Zempleni J, Mock DM. The efflux of biotin from human peripheral blood mononuclear cells. J Nutr Biochem **10**:105–109, 1999.
6. Zempleni J, Mock DM. Human peripheral blood mononuclear cells: Inhibition of biotin transport by reversible competition with pantothenic acid is quantitatively minor. J Nutr Biochem **10**:427–432, 1999.
7. Zempleni J, Mock DM. Mitogen-induced proliferation increases biotin uptake into human peripheral blood mononuclear cells. Am J Physiol **276**:C1079–C1084, 1999.
8. Zempleni J, Mock DM. Utilization of biotin in proliferating human lymphocytes. J Nutr **130**:335S–337S, 2000.
9. Zempleni J, Mock DM. Lymphocytes increase biotin uptake during G_1 phase of the cell cycle to increase biotinylation of histones. (abstract) FASEB J **14**:A243, 2000.
10. Aw TY, Jones DP, McCormick DB. Uptake of riboflavin by isolated rat liver cells. J Nutr **113**:1249–1254, 1983.
11. Dyer DL, Said HM. Riboflavin uptake by native *Xenopus laevis* oocytes. Biochim Biophys Acta **1234**:15–21, 1995.
12. Said HM, McCloud E, Yanagawa N. Riboflavin uptake by rat liver basolateral membrane vesicles. Biochim Biophys Acta **1236**:244–248, 1995.
13. Kumar CK, Yanagawa N, Ortiz A, Said HM. Mechanism and regulation of riboflavin uptake by human renal proximal tubule epithelial cell line HK-2. Am J Physiol **274**:F104–F110, 1998.
14. Said HM, Ortiz A, Ma TY, McCloud E. Riboflavin uptake by the human-derived liver cells HepG2: Mechanism and regulation. J Cell Physiol **176**:588–594, 1998.
15. Chastain JL, McCormick DB. Flavin catabolites: Identification and quantitation in human urine. Am J Clin Nutr **46**:830–834, 1987.
16. Chastain JL, McCormick DB. Clarification and quantitation of primary (tissue) and secondary (microbial) catabolites of riboflavin that are excreted in mammalian (rat) urine. J Nutr **117**:468–475, 1987.
17. Chastain JL, McCormick DB. Characterization of a new flavin metabolite from human urine. Biochim Biophys Acta **967**:131–134, 1987.
18. Mock DM. Biotin. In: Ziegler EE, Filer LJ Jr., Eds. Present Knowledge in Nutrition (7th ed). Washington, DC: International Life Sciences Institutes—Nutrition Foundation, pp220–235, 1996.
19. Zempleni J, Mock DM. Biotin biochemistry and human requirements. J Nutr Biochem **10**:128–138, 1999.
20. Yates AA, Schlicker SA, Suitor CW. Dietary reference intakes: The new basis for recommendations for calcium and related nutrients, B vitamins, and choline. J Am Diet Assoc **98**:699–706, 1998.

21. Rivlin RS. Hormonal regulation of riboflavin metabolism. In: Rivlin RS, Ed. Riboflavin. New York: Plenum Press, pp393–426, 1975.
22. Lee S-S, McCormick DB. Thyroid hormone regulation of flavocoenzyme biosynthesis. Arch Biochem Biophys **237**:197–201, 1985.
23. Boyum A. Isolation of mononuclear cells and granulocytes from human blood: Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1*g*. Scand J Clin Lab Invest **21**:77–89, 1968.
24. Zempleni J, Galloway JR, McCormick DB. Pharmacokinetics and utilization of orally and intravenously administered riboflavin in healthy humans. Am J Clin Nutr **63**:54–66, 1996.
25. Zar JH. Biostatistical Analysis. Englewood Cliffs, NJ: Prentice-Hall, Inc., 1974.
26. Abacus Concepts. SuperANOVA. Berkeley, CA: Abacus Concepts, Inc., 1989.
27. Steen HB, Nielsen V. Lymphocyte blastogenesis studied by volume spectroscopy. Scand J Immunol **10**:135–143, 1979.
28. Williams GT, Lau KMK, Coote JM, Johnstone AP. NAD metabolism and mitogen stimulation of human lymphocytes. Exp Cell Res **160**: 419–426, 1985.
29. Porsche AM, Kürber C, Englich S, Hartmann U, Rau G. Determination of the permeability of human lymphocytes with a microscope diffusion chamber. Cryobiology **23**:302–316, 1986.
30. Innis WS, McCormick DB, Merrill AH Jr. Variations in riboflavin binding by human plasma: Identification of immunoglobulins as the major proteins responsible. Biochem Med **34**:151–165, 1985.