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Biotin homeostasis during the cell cycle

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Peripheral blood mononuclear cells (PBMC) accumulate biotin by a Na-dependent energy-requiring transporter. This transporter might be the so-called Na-dependent multivitamin transporter, but kinetic observations suggest the existence of a second, more specific, biotin transporter. PBMC respond to proliferation by increased uptake of biotin; the increase is probably mediated by an increased number of transporters on the cell surface. The inferred increase in the biotin transporter synthesis is relatively specific. The increased uptake of biotin into proliferating PBMC is consistent with the hypothesis that these cells have an increased demand for biotin. Indeed, proliferating PBMC increase expression of genes encoding β-methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase, generating a quantitatively significant increased demand for biotin as a coenzyme in newly-synthesized carboxylases. Moreover, expression of the holocarboxylase synthetase gene increases, consistent with the synthesis of new holocarboxylases. In addition, proliferating PBMC increase both the density of biotinylation of histones and the mass of biotinylated histones per cell, suggesting a potential role for biotin in transcription and replication of DNA.

Biotin: Cell cycle: Peripheral blood mononuclear cells: Na-dependent multivitamin transporter

The mammalian cell cycle

The cell cycle is an ordered set of processes by which one cell grows and divides into two daughter cells (Murray & Hunt, 1993). Two fundamentally different cell cycles exist in mammals: the mitotic cell cycle, in which two diploid daughter cells are generated; the meiotic cell cycle, in which progeny cells are produced that contain half as many chromosomes as their

Abbreviations: cGMP, cyclic guanosine monophosphate; K_m, Michaelis–Menton constant; PBMC, peripheral blood mononuclear cells; SMVT, Na-dependent multivitamin transporter.

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parents (generation of sperm and egg). The present review will focus exclusively on the mitotic cell cycle of non-reproductive cells.

Absence of certain growth factors causes cells to enter a specialized resting state termed G0 (Murray & Hunt, 1993); cells in G0 do not divide (Fig. 1). Cells are normally recruited into the cell cycle by endogenous factors (e.g. hormones) and exogenous factors (e.g. antigens); they also can be recruited into the cell cycle by stimulation with specific exogenous factors (e.g. mitogens). The mitotic cell cycle can be thought of as occurring in two major sections, interphase (which includes phases G1, S and G2) and mitosis. The cycle ends with the division of the cell.

Interphase

The interphase is composed of three phases (Murray & Hunt, 1993). During the G1 phase processes such as synthesis of membranes, mitochondria and most cellular proteins occur. During the S phase, DNA for chromosome replication is synthesized. On completion of the S phase, cells enter the G2 phase in which growth processes prepare the cell for mitosis ('M').

Mitosis

Mitosis is subdivided into four phases (Murray & Hunt, 1993). During the first phase (prophase), the chromosomes condense (Fig. 2). In prometaphase the membrane of the nucleus breaks down, and the chromosomes attach to microtubules which are connected to centrosomes. In metaphase the microtubule-bound chromosomes line up midway between the two centrosomes. Dissolution of the linkage between sister chromatids marks the beginning of anaphase; sister chromatids separate and move away from each other to opposite poles of the spindle. Finally, cytokinesis (the physical process of cell division) begins.

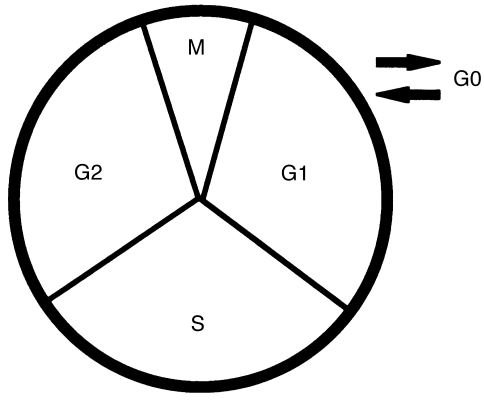

Fig. 1. The cell cycle. The sections approximately parallel the percentage duration of the phases of the cell cycle. G0, resting state; G1, gap 1 phase; S, DNA synthesis; G2, gap 2 phase; M, mitosis.

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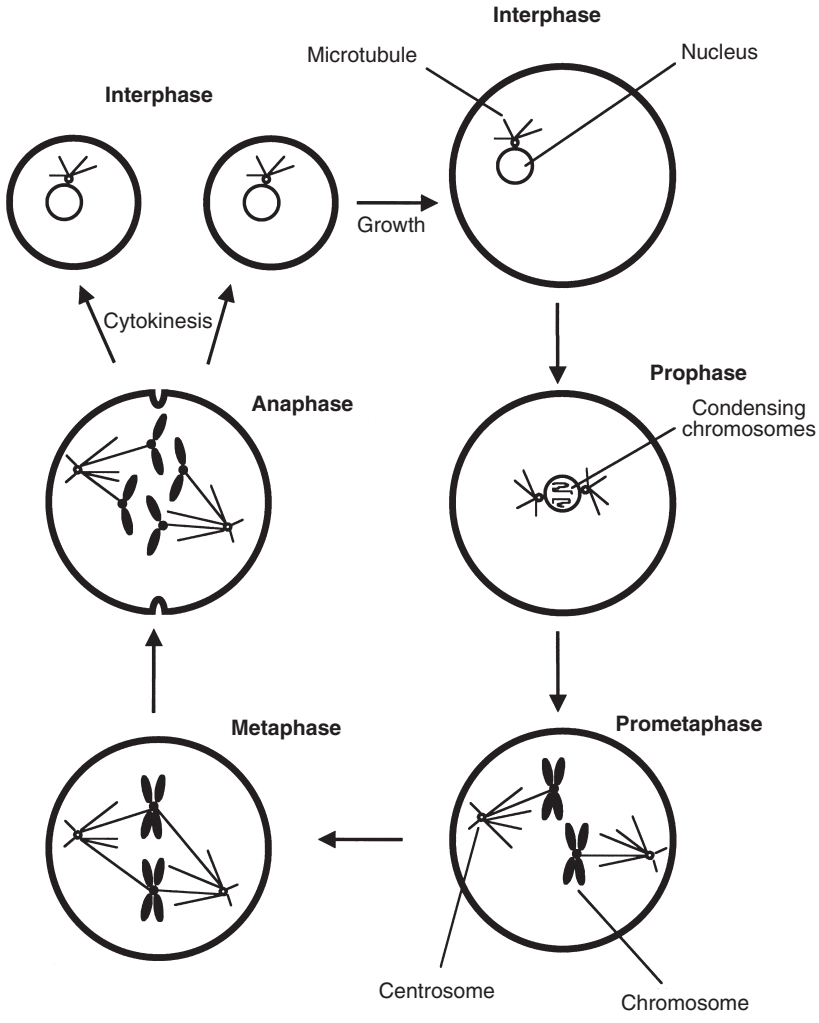

Fig. 2. Diagrammatic representation of the four phases of mitosis.

Nutrient supply may affect cell proliferation

Nutrient supply may affect cell proliferation by any of the following mechanisms: (1) nutrients are needed to provide metabolic energy (e.g. glucose); (2) nutrients are utilized in biosynthetic pathways (e.g. amino acids for protein synthesis); (3) nutrients are utilized as coenzymes (e.g. water-soluble vitamins); (4) nutrients directly affect gene expression or DNA synthesis (e.g. folic acid). For example, lymphocytes respond to mitogens by increasing uptake of alanine, proline and leucine (Segel & Lichtman, 1981), and by increasing the rate of glucose utilization (Roos *et al.* 1972; Loos & Roos, 1973; Roos & Loos, 1973). In mitogen-stimulated lymphocytes, fourteen amino acids are essential to maintain normal rates of protein synthesis and proliferation (Waithe *et al.* 1975). Likewise, the micronutrient linoleic acid is required to support maturation of B lymphocytes to immunoglobulin-secreting cells (Lernhardt, 1990).

Deficiency of nutrients often causes arrest of cells in the G0 phase; cells do not enter the

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cell cycle and do not divide. For example, HeLa cells arrest in the G0 phase if incubated in serine-free or biotin-free medium (Dakshinamurti *et al.* 1985). Taken together, these findings are consistent with the hypothesis that proliferating cells have nutrient demands that are different from those of non-proliferating quiescent cells.

Essential roles of biotin in carboxylases, gene expression, biotinylation of histones, and cell proliferation

Various physiological roles of biotin have been identified; these roles are probably the basis for the role of biotin in cell proliferation. In intermediary metabolism, biotin is an essential coenzyme for carboxylases. Biotin affects gene expression, and biotin is used to biotinylate histones. These roles of biotin will now be discussed.

Biotin-dependent carboxylases

The best characterized role of biotin in metabolism is as a covalently-bound coenzyme in carboxylases. The required covalent attachment of biotin to the ε-amino group of lysine in each of the apocarboxylases is catalysed by holocarboxylase synthetase (Dakshinamurti & Chauhan, 1994). Four biotin-dependent carboxylases have been identified in mammals (Fig. 3). These are acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase, and β-methylcrotonyl-CoA carboxylase (Wood & Barden, 1977; Knowles, 1989). Acetyl-CoA carboxylase is located both in cytosol and in mitochondria (Kim, 1997). Acetyl-CoA carboxylase catalyses the binding of bicarbonate to acetyl-CoA to form malonyl-CoA; the latter is a substrate of fatty acid synthesis. Acetyl-CoA carboxylase may also play a role in biotin storage (Shriver & Allred, 1990; Shriver *et al.* 1993).

Pyruvate carboxylase, propionyl-CoA carboxylase, and β-methylcrotonyl-CoA carboxylase are located in mitochondria. Pyruvate carboxylase is a key enzyme in gluconeogenesis and anapleurosis of a tricarboxylic acid cycle intermediate. Propionyl-CoA carboxylase catalyses an essential step in the metabolism of isoleucine, valine, methionine, threonine, the cholesterol side chain and odd-chain fatty acids. β-Methylcrotonyl-CoA carboxylase catalyses an essential step in leucine metabolism. If cell proliferation increases demand for one or more of the intermediates that are synthesized in pathways dependent on carboxylases (e.g. malonyl-CoA in order to increase synthesis of fatty acids for new cell membranes), one would predict an increased demand for biotin as a coenzyme.

Proteolytic degradation of holocarboxylases leads to the formation of biotinyl peptides, including biocytin (biotinyl-ε-lysine). Biotinidase releases biotin from oligopeptides and biocytin for recycling into new holocarboxylases (Wolf *et al.* 1985).

Gene expression

Evidence has been provided that biotin potentially plays a role in gene expression. In pioneering studies Dakshinamurti & Cheah-Tan (1968) demonstrated that biotin deficiency in rats causes a 40–45 % reduction of liver glucokinase activity; enzyme activity can be restored to normal by biotin administration (Dakshinamurti & Cheah-Tan, 1968). Further, biotin administration to biotin-deficient rats increases the synthesis rate of protein, rRNA, and mRNA

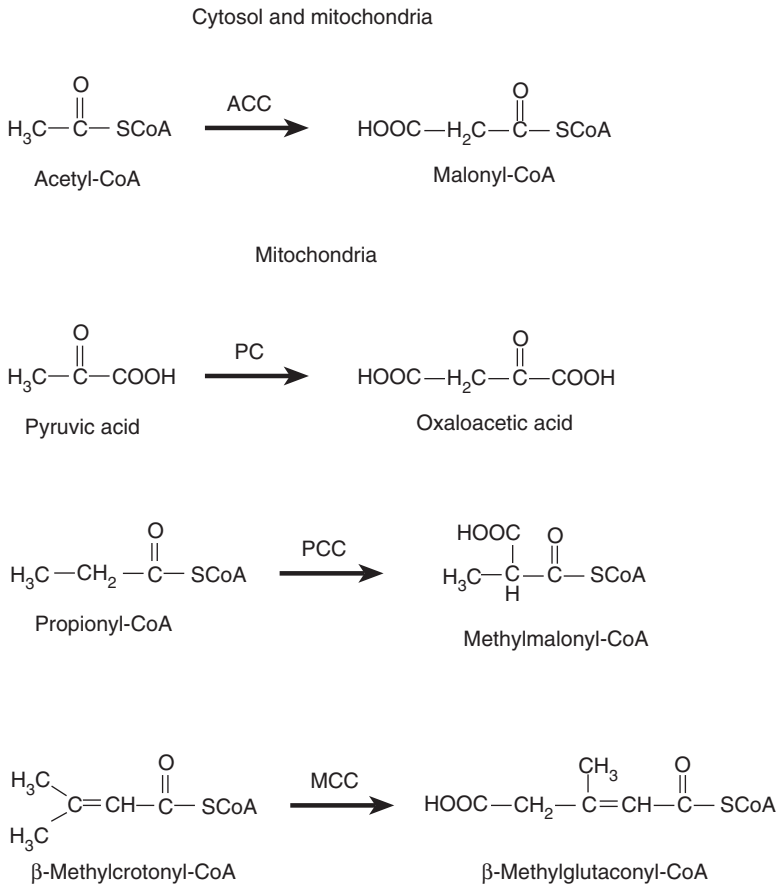

Fig. 3. Biotin-dependent carboxylases. ACC, acetyl-CoA carboxylase; PC, pyruvate carboxylase; PCC, propionyl-CoA carboxylase; MCC, β-methylcrotonyl-CoA carboxylase.

(Dakshinamurti & Litvak, 1970). Spence & Koudelka (1984) and Chauhan & Dakshinamurti (1991) demonstrated that administration of biotin increases the amount of mRNA coding for glucokinase in rat liver. Biotin also increases both mRNA coding for glucokinase and glucokinase activity in cultured beta cells (Borboni *et al.* 1996).

Biotin may also repress expression of certain genes. For example, administration of biotin to diabetic rats repressed hepatic phospho*enol*pyruvate carboxykinase mRNA to 15 % of the control levels (Dakshinamurti & Chauhan, 1994). Moreover, biotin deficiency is associated with decreased ornithine transcarbamylase activity and mRNA in rat liver (Maeda *et al.* 1996).

Hypothetically, the effects of biotin on gene expression might be mediated by cyclic guanosine monophosphate (cGMP). Pharmacological concentrations of biotin cause increased concentrations of cellular cGMP (Vesely, 1982; Vesely *et al.* 1984), a known activator of RNA polymerase I and, hence, rRNA synthesis (Johnson & Hadden, 1975). The time courses of cellular cGMP, mRNA coding for glucokinase and glucokinase activity support the idea of a biotin-initiated and cGMP-mediated sequence of gene expression. Addition of biotin to the

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culture medium of rat hepatocytes increases cGMP levels maximally within 1 h, mRNA levels maximally after 2 h, and glucokinase activity maximally after 6 h (Spence & Koudelka, 1984). These studies have in common the fact that non-physiological high concentrations of biotin were used, suggesting a pharmacological effect of biotin.

Alternatively to its effect on mRNA synthesis (transcription), biotin might affect the intracellular concentration of some proteins on the post-transcriptional level. If HepG2 cells are grown in biotin-deficient medium, expression of the asialoglycoprotein receptor is reduced under conditions where protein synthesis, total cellular protein content and mRNA coding for asialoglycoprotein receptor are comparable with those of control cells; addition of biotin or biocytin restores receptor expression (Collins *et al.* 1988). These findings are consistent with the hypothesis that a biotin-dependent post-transcriptional event permits the ultimate expression of asialoglycoprotein receptor by HepG2 cells. Analogous studies of propionyl-CoA carboxylase in rat hepatocytes suggest that biotin regulates the expression of this carboxylase also at a post-transcriptional step (Rodriguez-Melendez *et al.* 1999).

Taken together, these studies provide evidence for a role of biotin in gene expression and post-translational processing of proteins, although mechanisms directly linking the biotin molecule to its effects are lacking. These findings provide a background for the hypotheses that biotin is involved in cell cycle regulation and that biotin deficiency may adversely affect cell growth.

Biotinylation of histones

Recently, Hymes *et al.* (1995*a*,*b*) discovered that human biotinidase has biotinyl transferase activity, in which biocytin serves as a biotin donor and histones serve as specific biotin acceptors. Histones play an important role in the regulation of transcription, replication and packaging of DNA (Wolffe, 1998). Gene expression as well as DNA condensing during mitosis are affected by reversible chemical modification of the histones; for example, acetylation, methylation, phosphorylation or ADP-ribosylation of histones (Kaye & Sheratzky, 1969; Paik & Kim, 1969; Langan, 1970; Hohmann, 1983; Ausio & van Holde, 1986; Boulikas, 1988; Hebbes *et al.* 1988; Boulikas *et al.* 1990; Roth & Allis, 1992; Lee *et al.* 1993; Sommerville *et al.* 1993). These observations raise the possibility that biotinylation of histones might serve as a mechanism through which biotin status affects gene expression.

Although the physiological role of biotinylated histones is far from clear, the following observations are consistent with a role for biotin in modifying histones, and in turn affecting the packaging of DNA: (1) biotinidase is ubiquitous in mammalian cells and 25 % of the cellular biotinidase activity is located in the nuclear fraction (Pispa, 1965); (2) histones dissociate from the DNA in biotin-deficient rats (Petrelli *et al.* 1976); (3) biotin deficiency in rats results in decreased phosphorylation and methylation of histones as well as increased acetylation of histones (Petrelli *et al.* 1978); (4) biotin-rich nuclei in neoplastic tissue exhibit a unique arrangement of chromatin substructures (Nakatani *et al.* 1994).

Taken together, these observations suggest an important role for biotin in modification of histones, leading to potential effects on transcription and replication of DNA. Since cell proliferation requires a substantial increase of both replication and transcription, an increased uptake of biotin by proliferating cells to biotinylate histones is a plausible mechanism by which this nutrient exerts at least some of its effects on cell proliferation.

Cell proliferation

Biotin is essential for cell proliferation. For example, incubation of HeLa cells in biotin-free medium causes arrest in the G0 phase of the cell cycle (Dakshinamurti *et al.* 1985). Studies in our laboratory indicate that the proliferation rate of human lymphocytes depends on a sufficient biotin supply (J Zempleni and DM Mock, unpublished results). Biotin also stimulates the production of an unidentified growth factor in cells (Moskowitz & Cheng, 1985).

Effects of biotin on proliferation of immune cells can be clinically relevant in some circumstances. For example, biotin deficiency caused by omission of biotin from total parenteral nutrition is associated with cutaneous fungal infection (Mock, 1996). Inborn errors of biotin metabolism cause impaired humoral and cellular immunity in humans and a tendency towards cutaneous fungal infections (Cowan *et al.* 1979).

Peripheral blood mononuclear cells as a cell model

The observations described provide information that cell proliferation may affect biotin homeostasis (and vice versa), as discussed earlier. However, limited information is available concerning the mechanism(s). The following sections will provide the first in-depth review of recent studies that have directly addressed the interaction between cell proliferation and biotin metabolism. Most of these studies arise from our laboratory and use peripheral blood mononuclear cells (PBMC) as a cell model. PBMC represent a heterogeneous population of immune cells (B-cells, T-cells, and various granulocytes) that arise from pluripotent haematopoietic stem cells in the bone marrow (Janeway *et al.* 1999). PBMC were chosen as a model for the following reasons: (1) PBMC are human cells and therefore results directly apply to human nutrition; (2) PBMC are easily obtainable from peripheral blood without organ biopsy; (3) PBMC have been used successfully as models in nutrition research (for example, biotin status can be assessed by measuring activities of biotin-dependent carboxylases in PBMC (Velazquez *et al.* 1990, 1995), and vitamin transport has been studied in PBMC (Zempleni & Mock, 1998, 1999*a*, 1999*b*; 2000*b*)); (4) freshly-isolated PBMC have a full set of chromosomes that are not deranged, unlike in many immortalized cell lines; (5) freshly-isolated PBMC are typically quiescent, and some PBMC (B- and T-cells) will reliably proliferate rapidly after antigenic or mitogenic stimulation.

Biotin transport in quiescent peripheral blood mononuclear cells

Mechanisms and rates of biotin uptake and efflux in quiescent PBMC have been investigated extensively in order to establish a model of cellular biotin metabolism. Biotin uptake into PBMC is a transporter-mediated process that requires metabolic energy (Zempleni & Mock, 1998). Biotin is cotransported with Na; cellular uptake depends on Na–K–ATPase. Biotin uptake into other cells is mediated by a similar mechanism, for example, in rat liver cells (Bowers-Komro & McCormick, 1985) and HepG2 cells (Said *et al.* 1994). At a physiological concentration of biotin in the medium (475 pmol/l), the rate of biotin uptake into quiescent PBMC (450 (SD 34) amol/(10^6 cells $\times$ 30 min)) is approximately one-third the uptake rate of the vitamin riboflavin (Zempleni & Mock, 1998, 2000*b*).

The biotin transporter in PBMC is structurally specific for biotin. The thiophane portion of the biotin molecule is important for binding by the biotin transporter in PBMC (Zempleni &

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Mock, 1998). This conclusion is based on the observation that dethiobiotin (a biotin analogue with modified thiophane ring) competes for cellular biotin uptake much less efficiently than biotin analogues with an intact thiophane ring. Compounds that are structurally similar to biotin but are not biotin analogues (lipoic acid, pantothenic acid, hexanoic acid, bilirubin) do not compete with biotin for cellular uptake (Zempleni & Mock, 1998, 1999*b*). This finding apparently contrasts with properties of the Na-dependent multivitamin transporter (SMVT). This mammalian transporter binds biotin, pantothenic acid, and lipoic acid with similar affinities (Prasad *et al.* 1997; Wang *et al.* 1999). Although we have demonstrated the existence of SMVT in PBMC (J Zempleni, JS Stanley and DM Mock, unpublished results), our studies of substrate specificity and transport kinetics suggest that a second, more specific, biotin transporter is responsible for biotin transport in PBMC. Alternatively, protein–protein interactions between SMVT and PBMC-specific protein on the cell surface might account for the differences in substrate specificity and Michaelis–Menten constant (K_m) we have observed.

The transporter that mediates biotin uptake into PBMC also mediates biotin efflux (classic 'countertransport'). This conclusion is based on studies in which [^{3}H]biotin-loaded PBMC were transferred into a medium that contained either unlabelled biotin or biotin analogues; controls were studied in a biotin-free medium (Zempleni & Mock, 1999*a*). [^{3}H]biotin efflux was significantly greater in the presence of either biotin or a biotin analogue compared with that in biotin-free medium. This finding is consistent with the hypothesis that intracellular [^{3}H]biotin leaves PBMC in exchange for extracellular biotin via countertransport.

[^{3}H]biotin efflux data from human PBMC were used to model the elimination kinetics of biotin at the cellular level (Zempleni & Mock, 1999*a*). Biotin efflux from PBMC at 37˚C is fast and triphasic; the half-lives of the three elimination phases are approximately 0·2, 1·2 and 22 h. It is likely that the half-life of [^{3}H]biotin efflux during the terminal slow phase of efflux is determined by the breakdown of biotin-dependent carboxylases to release free [^{3}H]biotin, and the subsequent efflux of [^{3}H]biotin from PBMC. The half-life of [^{3}H]biotin during the terminal phase of efflux (22 h) resembles the half-lives that have been measured for pyruvate carboxylase (28 h) and acetyl-CoA carboxylase (4·6 d; Majerus & Kilburn, 1969; Nakanishi & Numa, 1970; Weinberg & Utter, 1979, 1980; Freytag & Merton, 1983).

Effects of cell proliferation on biotin uptake

Rates of biotin uptake

Mitogen-stimulated proliferating PBMC accumulate biotin at a rate five times faster than unstimulated controls (Zempleni & Mock, 1999*c*). For example, when proliferation was induced by incubation with pokeweed mitogen (lectin from *Phytolacca americana*) for 3 d, biotin uptake increased in dose-dependent fashion from 481 % to 722 % of the control value (Fig. 4). When proliferation was induced by either concanavalin A or phytohaemagglutinin, a similar increase in biotin uptake was observed.

The time course of transport induction is consistent with the hypothesis that increased biotin transport into mitogen-induced PBMC is caused by increased biotin demand due to proliferation. Stimulation of biotin uptake is maximal 48–72 h after addition of pokeweed mitogen, which coincides with the maximal rate of proliferation (Zempleni & Mock, 1999*c*). Moreover, short-term incubation (<15 min) of PBMC with mitogens did not alter transport rates of biotin, suggesting that mitogens do not directly increase biotin transport; rather, the effects of mitogen stimulation are mediated by induction of cell proliferation.

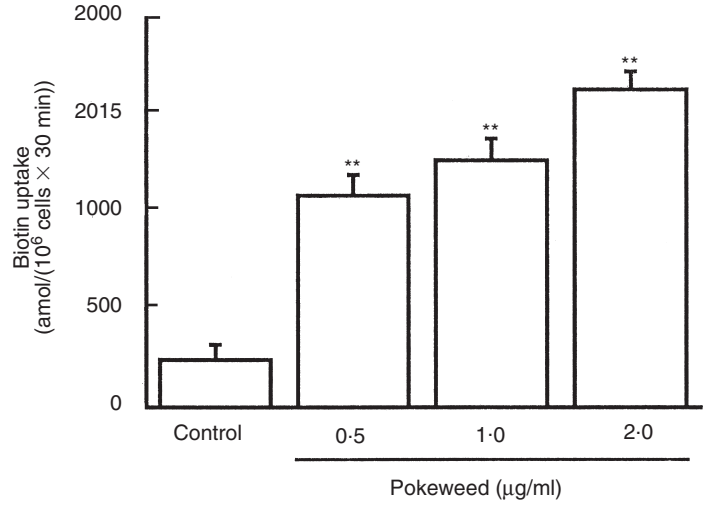

Fig. 4. Uptake of [^{3}H]biotin into proliferating human peripheral blood mononuclear cells and quiescent controls. Proliferation was induced by incubation with pokeweed (*Phytolacca americana*) lectin (0·5, 1·0, or 2·0 μg/ml) at 37°C for 3 d; controls were incubated without pokeweed lectin. Values are means and 1 SD represented by vertical bars for six determinations. Mean values were significantly different from those for the controls (ANOVA with Dunnett's procedure): $^{**}P < 0{\cdot}01$. (From Zempleni & Mock, 1999*c*.)

Biotin uptake at specific phases of the cell cycle

The studies described earlier provide evidence that PBMC respond to proliferation by an increased uptake of biotin. However, these studies were conducted using non-synchronized cultures of PBMC, i.e. mixed populations of cells from various phases of the cell cycle. We proceeded to identify those phases of the cell cycle during which biotin uptake is increased. These pilot studies are summarized here (Zempleni & Mock, 2000*a*). PBMC were recruited into the cycle (G0 to G1 transition) by incubation with concanavalin A (20 μg/ml) for 36 h; PBMC to be studied in the G0 phase of the cycle (i.e. quiescent cells) were not exposed to concanavalin A. Then, the PBMC were arrested at designated phases of the cell cycle by incubation overnight at 37°C with one of the following chemicals: cyclosporin A (300 nmol/l) to cause G0 arrest; wortmannin (100 nmol/l) to cause G1 arrest; aphidicolin (118 μmol/l) to cause arrest at the G1/S interphase; colchicine (5 μmol/l) to cause M arrest. Biotin uptake rates into these cells were measured using [^{3}H]biotin.

Biotin uptake rates were significantly different among the phases of the cell cycle (Fig. 5). PBMC arrested in the G1, S, or M phases transported biotin about twice as fast as PBMC arrested in the G0 phase. Transport rates in G0-arrested cells were not significantly different from quiescent cells without chemical G0 arrest (data not shown). The fact that biotin transport increases as early as G1 and remains increased through later phases of the cell cycle provides evidence that the number of biotin transporters increases early in the course of proliferation.

Transport kinetics

Theoretically, PBMC can increase biotin uptake either by increasing the number of biotin transporters on the cell surface or by increasing substrate affinity of biotin transporters (or both). To

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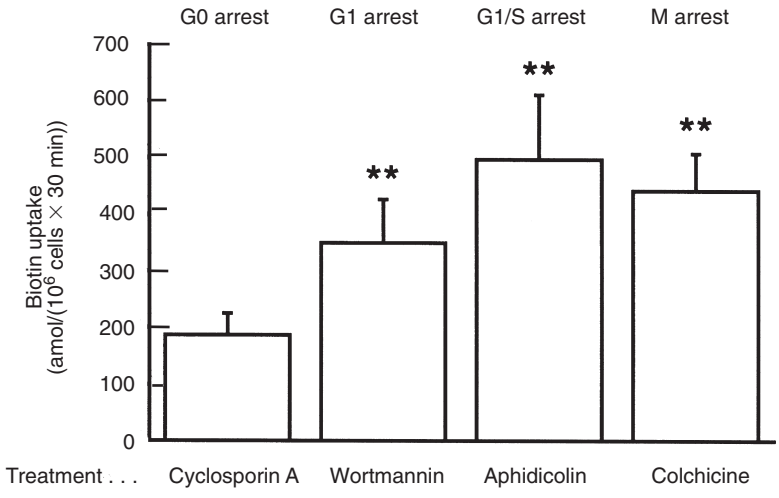

Fig. 5. Uptake of [^{3}H]biotin into cell-cycle arrested human peripheral blood mononuclear cells. For details of procedures, see p. 51. Values are means and standard deviations represented by vertical bars for six determinations. Mean values were significantly different from those for the controls: $^{**}P < 0{\cdot}01$. (From Zempleni & Mock, 2000*a*.)

determine which of the two mechanisms mediates the increased uptake of biotin, the transport kinetics of biotin uptake were compared in pokeweed-stimulated PBMC and quiescent controls; biotin concentrations in the medium were set at various levels from 238 to 2850 pmol/l. Lineweaver–Burk plots of the uptake data revealed intersection near the *x*-axis for the regression lines of proliferating and quiescent PBMC (Fig. 6). The maximal transport rates of the biotin transporter in proliferating PBMC and in controls were 9·1 (SD 6·6) and 2·3 (SD 1·6) fmol/(10^6 cells × 30 min) respectively ($P < 0{\cdot}05$). The K_m of the biotin transporter in proliferating PBMC and in controls were 2·4 (SD 1·7) and 3·7 (SD 3·4) nmol/l, respectively ($P = 0{\cdot}86$, not significant). These findings suggest that increased biotin uptake is mediated by an increased number of biotin transporters on the cell surface rather than by an increased substrate affinity of biotin transporters.

Transporter specificity

We considered that increased biotin uptake is mediated by increased synthesis of a transporter other than the specific biotin transporter described in the quiescent PBMC (Zempleni & Mock, 1998); for example, the SMVT described earlier (Prasad *et al.* 1997; Wang *et al.* 1999). The SMVT exhibits competitive inhibition among biotin, pantothenic acid and lipoic acid. Potentially, proliferating PBMC might synthesize more of the SMVT rather than the specific biotin transporter.

To investigate this possibility, the effect of lipoic acid on biotin transport was measured in proliferating and quiescent cells. Lipoic acid is a competitive inhibitor of biotin transport into various other cell types (Said *et al.* 1994; Prasad *et al.* 1998). Extracellular lipoic acid does not significantly reduce [^{3}H]biotin uptake into quiescent PBMC (Fig. 7; Zempleni & Mock, 1999*c*), confirming results from previous studies (Zempleni & Mock, 1998). Likewise in proliferating PBMC, lipoic acid does not inhibit biotin uptake (Zempleni & Mock, 1999*c*). [^{3}H]Biotin uptake

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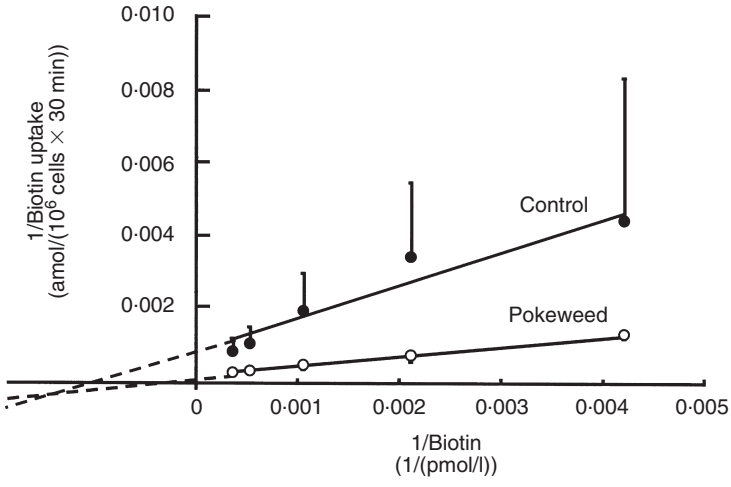

Fig. 6. Lineweaver–Burk plot of biotin uptake into proliferating human peripheral blood mononuclear cells (PBMC) and quiescent controls. PBMC were incubated with pokeweed (*Phytolacca americana*) lectin (2·0 μg/ml; ○) at 37°C for 3 d to induce proliferation; controls (●) were incubated without pokeweed lectin. Biotin concentration in the medium ranged from 238 to 2850 pmol/l. Values are means and 1 SD represented by vertical bars for three determinations. (From Zempleni & Mock, 1999*c*.)

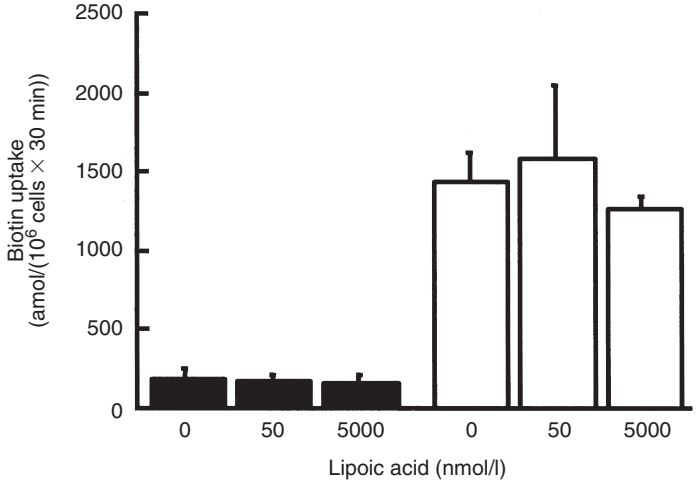

Fig. 7. Uptake of [^{3}H]biotin into proliferating and quiescent human peripheral blood mononuclear cells (PBMC) in the presence of DL-α-lipoic acid. PBMC were incubated with pokeweed (*Phytolacca americana*) lectin (2·0 μg/ml) at 37°C for 3 d to induce proliferation (□); quiescent PBMC were incubated without pokeweed lectin (■). PBMC were then incubated with [^{3}H]biotin (475 pmol/l) and lipoic acid (either 50 or 5000 nmol/l; controls included no lipoic acid) at 37°C for 30 min. Values are means and 1 SD represented by vertical bars for six determinations. (From Zempleni & Mock, 1999*c*.)

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in the presence of physiological (50 nmol/l) or pharmacological (5000 nmol/l) concentrations of lipoic acid was 113 (SD 47) % and 89 (SD 11) % respectively, of uptake in lipoic acid-free medium ($P = 0{\cdot}15$; not significant). If the SMVT were responsible, we would have expected a significant decrease in biotin transport in the presence of pharmacological concentrations of lipoic acid. These data provide evidence that the transporter in both proliferating and quiescent PBMC is the structurally-specific biotin transporter. Moreover, the finding that K_m values for biotin transport are similar in proliferating and quiescent PBMC (earlier, p. 52) provides additional evidence that the same transporter accounts for biotin uptake in both cells.

What drives the increased uptake of biotin in proliferating cells?

Carboxylases

Theoretically, cells might respond to proliferation by increasing activities of (some) carboxylases in order to synthesise intermediates for anabolic pathways or to degrade intermediates in catabolic pathways. Thus, recent studies have investigated activities of the four biotin-dependent carboxylases in proliferating PBMC (Zempleni & Mock, 2000*c*). In these studies proliferation of human PBMC was stimulated by incubation with either pokeweed lectin or concanavalin A for 3 d; controls were incubated without mitogen. At days 1, 2 and 3 after addition of mitogen, activities of β-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase, acetyl-CoA carboxylase, and pyruvate carboxylase were determined. At 3 d after addition of pokeweed lectin, the activity of β-methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase had increased by 180 and 50 % respectively, compared with time zero (before pokeweed lectin; Fig. 8). Values in concanavalin A-stimulated PBMC were similar (data not shown).

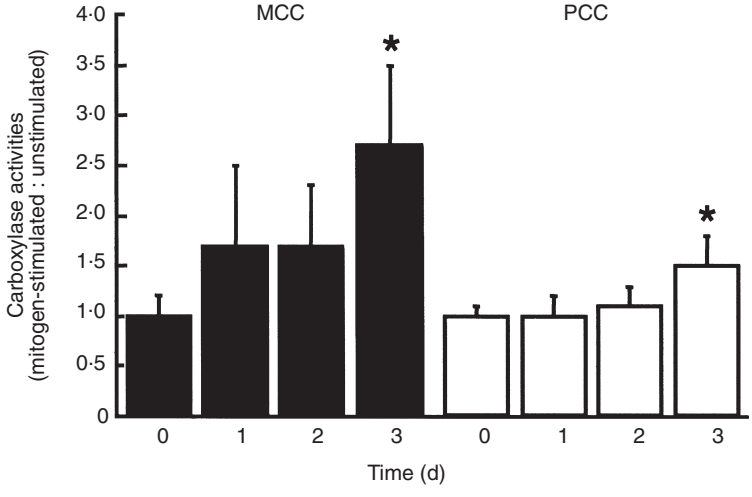

Fig. 8. Activities of biotin-dependent carboxylases in pokeweed (*Phytolacca americana*) lectin-stimulated human peripheral blood mononuclear cells (PBMC). PBMC were incubated with pokeweed lectin (2·0 μg/ml) for the indicated times. The carboxylase activities were measured at timed intervals. (■), β-Methylcrotonyl-CoA carboxylase (MCC); (□), propionyl-CoA carboxylase (PCC). Values are means and 1 SD represented by vertical bars for six determinations. Mean values were significantly different from those for the controls (time zero): $^*P < 0{\cdot}05$. (From Zempleni & Mock, 2000*c*.)

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These data are consistent with the hypothesis that increased carboxylase activities in proliferating PBMC generate an increased requirement for the coenzyme biotin. Acetyl-CoA carboxylase and pyruvate carboxylase were not active enough to be detected.

Theoretically, increased carboxylase activities could be mediated either by biotinylation of apocarboxylases that are already present in cells or by increased biosynthesis of new apocarboxylases. In order to distinguish between these two mechanisms, expression rates of the genes encoding for β-methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase were measured (J Zempleni, JS Stanley and DM Mock, unpublished results); in addition, expression of the holocarboxylase synthetase gene was quantified (i.e. the protein that catalyses biotinylation of apocarboxylases). Levels of mRNA encoding β-methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase (alpha and beta chain), and holocarboxylase synthetase were 10–320 % greater in proliferating PBMC compared with quiescent controls. These findings are consistent with increased enzyme synthesis rather than with increased biotinylation of already-existing apocarboxylases.

Histones

If proliferating PBMC increase biotinylation of histones, this factor might contribute to an increased demand for biotin by proliferating PBMC. Our recent studies have investigated biotinylation of histones in proliferating and quiescent PBMC (Zempleni & Mock, 2000*a*). PBMC were incubated with either concanavalin A (20 μg/ml) or pokeweed lectin (2·0 μg/ml) for 3 d; controls were incubated without mitogen. Biotinylation of histones was tracked by adding [^{3}H]biotin at a physiological concentration (475 pmol/l) to the medium. After incubation, the PBMC were harvested by centrifugation, and the histones were extracted and purified. The [^{3}H]biotin-labelled histones were analysed by liquid-scintillation counting. Both quiescent and proliferating PBMC utilised biotin to biotinylate histones. Quiescent PBMC incorporated 0·07 (1 SD 0·05) fmol [^{3}H]biotin/10^6 cells into histones; concanavalin A-stimulated, proliferating PBMC incorporated 0·22 (1 SD 0·02) fmol [^{3}H]biotin/10^6 cells into histones (proliferating PBMC *v.* quiescent controls, $P < 0·05$); pokeweed lectin-stimulated PBMC incorporated 0·33 (1 SD 0·01) fmol [^{3}H]biotin/10^6 cells into histones (stimulated PBMC *v.* quiescent controls, $P < 0·05$). Moreover, the biotinylation stoichiometry (fmol biotin/μg histone) was greater in proliferating than in quiescent PBMC. Stoichiometry of histones increased from 0·16 (1 SD 0·11) fmol [^{3}H]biotin/μg histone in quiescent PBMC to 0·38 (1 SD 0·04) and 0·43 (1 SD 0·01) fmol [^{3}H]biotin/μg histone in concanavalin A- and pokeweed lectin-stimulated PBMC respectively (Fig. 9). These observations are consistent with the hypothesis that PBMC utilize biotin to biotinylate histones and that biotinylation of histones varies during the cell cycle.

Is the increase in biotin uptake specific?

We studied the cellular uptake of two other water-soluble vitamins (riboflavin and pantothenic acid) into proliferating PBMC in order to determine whether proliferating PBMC specifically increase biotin uptake. Riboflavin and pantothenic acid were chosen for the following reasons. First, riboflavin and biotin have no structural similarity, no known metabolic interaction and enter cells such as renal epithelial cells, liver cells and PBMC by different transport mechanisms (Kumar *et al.* 1998; Said *et al.* 1998; Zempleni & Mock, 2000*b*). Biotin uptake into PBMC is mediated by a Na-dependent transporter (Zempleni & Mock, 1998), whereas

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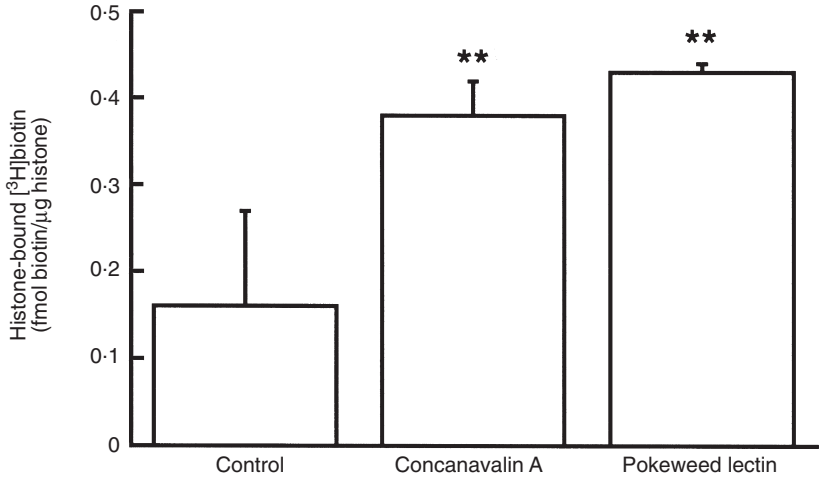

Fig. 9. Biotinylation of histones. Human peripheral blood mononuclear cells were incubated with pokeweed (*Phytolacca americana*) lectin (2·0 µg/ml) or concanavalin A (20 µg/ml) at 37°C for 3 d to induce proliferation; controls were incubated without mitogen. The medium contained 475 pmol [^{3}H]biotin/l. Histones were extracted and purified using hydrochloric acid washes, dialysis, and avidin chromatography. Values are means and 1 SD represented by vertical bars for four determinations. Mean values were significantly different from those for the control: $^{**}P < 0{\cdot}0$l. Note that total histones (H1, H2a, H2b, H3, and H4) were measured. (From Zempleni & Mock, 2000*a*.)

riboflavin uptake does not require Na (Zempleni & Mock, 2000*b*). Transport characteristics suggest that uptake of riboflavin is mediated by facilitated diffusion. Thus, riboflavin was chosen as a specificity control that does not interact with biotin at the cellular level. Second, pantothenic acid and biotin enter certain cells by sharing a common transporter, the SMVT (Prasad *et al.* 1997; Wang *et al.* 1999). Thus, the specificity control pantothenic acid provided additional insight into whether increased synthesis of SMVT by PBMC mediates increased uptake of both biotin and pantothenic acid.

Riboflavin

Using a physiological concentration of riboflavin in the medium, the time course of riboflavin uptake into proliferating PBMC was measured after addition of mitogen to the medium (Zempleni & Mock, 2000*b*). Proliferating PBMC increase riboflavin uptake in parallel with DNA synthesis (Fig. 10). However, for riboflavin proliferation was not associated with a change in maximal transport rate or K_m (Table 1), suggesting that some mechanism other than increased transporter synthesis or substrate affinity was responsible for increased uptake.

Our studies provided evidence that the increased riboflavin uptake was mediated by the increased cellular volume of proliferating PBMC (Zempleni & Mock, 2000*b*). The number of intracellular riboflavin molecules per PBMC increases during proliferation in proportion to the cell volume, whereas the cellular riboflavin concentration remains unchanged. Consistent with this hypothesis, we observed that osmotically-induced shrinkage of PBMC in hyperosmolar medium caused decreased riboflavin influx. Taken together, these studies suggest that prolifer-

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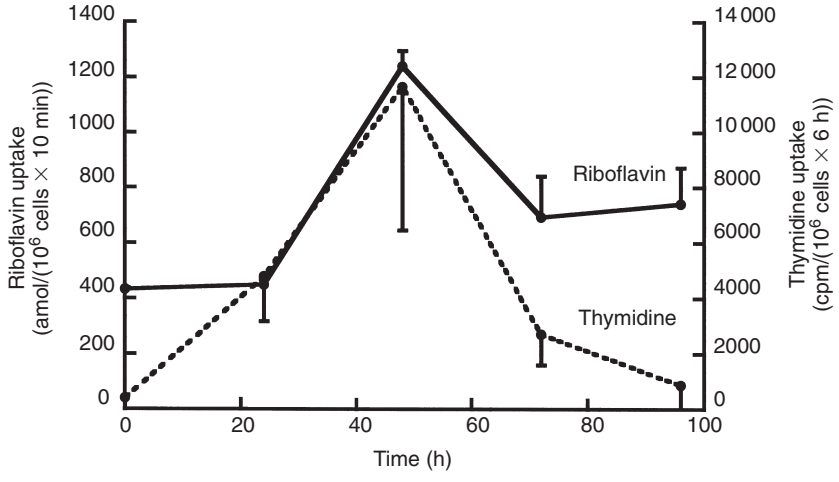

Fig. 10. Time course of [^{3}H]riboflavin and [^{3}H]thymidine uptake in concanavalin A-stimulated human peripheral blood mononuclear cells (20 μg concanavalin A/ml for up to 96 h). At timed intervals aliquots were collected and cellular uptake rates of [^{3}H]riboflavin (—) and [^{3}H]thymidine (-----) were measured. Zero-time values were measured before addition of concanavalin A to the medium. Values are means and 1 SD represented by vertical bars for five to six determinations. cpm, counts per min. (From Zempleni & Mock, 2000*b*.)

Table 1. Transport kinetics of riboflavin and pantothenic acid in mitogen-stimulated human peripheral blood mononuclear cells (PBMC) and quiescent controls*
(Mean values and standard deviations for five determinations for riboflavin and six to twelve determinations for pantothenic acid)

	Riboflavin				Pantothenic acid			
	V_{max}[a] (fmol/(10^6 cells × 10 min))		K_m (nmol/l)		V_{max} (pmol/(10^6 cells × 15 min))		K_m (μmol/l)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Concanavalin A	39	20	594	395	43	11	19	6·0
Pokeweed lectin	23	10	513	470	37	11	19	7·6
Control	28	19	537	375	7·5	3·3	13	6·7
Statistical significance of difference†: $P <$	NS		NS		0·01		NS	

V_{max}, maximal transport rate.
*PBMC were incubated with concanavalin A (20 μg/ml) or pokeweed lectin (2·0 μg/ml) at 37°C for 2 d (riboflavin) or 3 d (pantothenic acid) to induce proliferation; controls were incubated without mitogen. Then, vitamin uptake was measured using substrate concentrations in the medium that ranged from 5 to 1000 nmol [^{3}H]riboflavin/l and 50 to 20 000 nmol [^{3}H]pantothenic acid/l.
† Mitogen-stimulated PBMC *v.* control; NS, $P > 0·05$.

ating PBMC do not globally increase the synthesis of transporters for water-soluble vitamins. For some vitamins, increased influx is mediated by increased diffusion into cells in response to increased cell volume (Williams *et al.* 1985; Zempleni & Mock, 2000*b*).

Pantothenic acid

As with several other cell types (Prasad *et al.* 1997; Wang *et al.* 1999), PBMC probably accumulate pantothenic acid via the SMVT (J Zempleni, JS Stanley and DM Mock, unpublished

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results). SMVT also binds biotin, but the importance of SMVT for uptake of biotin into PBMC is uncertain (as noted earlier).

Using a physiological concentration of pantothenic acid in the medium, the time course of pantothenic acid uptake into proliferating PBMC was measured after addition of mitogen to the medium (J Zempleni, JS Stanley and DM Mock, unpublished results). PBMC respond to proliferation by an increase of pantothenic acid uptake (Fig. 11). Increased pantothenic acid uptake parallels proliferation rates, as judged by thymidine uptake; pantothenic acid uptake is stimulated maximally 48 h after addition of pokeweed lectin. With respect to biotin uptake, PBMC respond to proliferation by increasing the number of pantothenic acid transporters per cell, as judged by maximal transport rate (Table 1); substrate affinity remains unchanged, as judged by K_m. These findings suggest that proliferating PBMC increase the number of SMVT on the cell surface. Indeed, based on our studies, levels of mRNA encoding SMVT are approximately ten times greater in proliferating PBMC compared with quiescent controls (Fig. 12), consistent with an increased synthesis of SMVT. Moreover, the increase is relatively specific; the increased transcription of the SMVT gene exceeded the transcriptional increase in various housekeeping genes by at least sixfold (Fig. 12).

Taken together, the studies of pantothenic acid uptake provide evidence that the increased uptake of water-soluble vitamins observed in proliferating PBMC is not specific to biotin alone. Increased uptake of pantothenic acid and biotin probably occur by similar mechanisms. Determination of whether the increased uptake of both vitamins is mediated by the same transporter (SMVT) awaits further study.

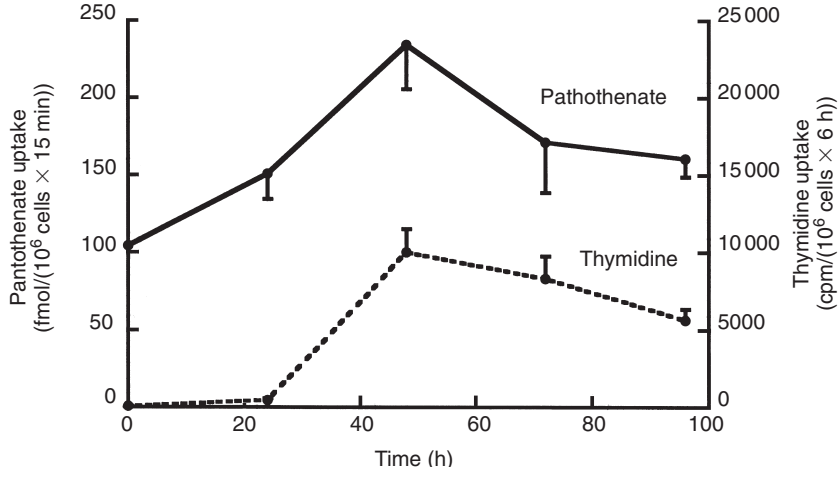

Fig. 11. Time course of [^{3}H]pantothenic acid and [^{3}H]thymidine uptake in pokeweed (*Phytolacca americana*) lectin-stimulated human peripheral blood mononuclear cells (2·0 μg pokeweed lectin/ml for up to 96 h). At timed intervals, aliquots were collected and cellular uptake rates of [^{3}H]pantothenic acid (—) and [^{3}H]thymidine (----) were measured. Zero-time values were measured before addition of concanavalin A to the medium. Values are means and 1 SD represented by vertical bars for six determinations. cpm, counts per min. (From J Zempleni, JS Stanley and DM Mock, unpublished results.)

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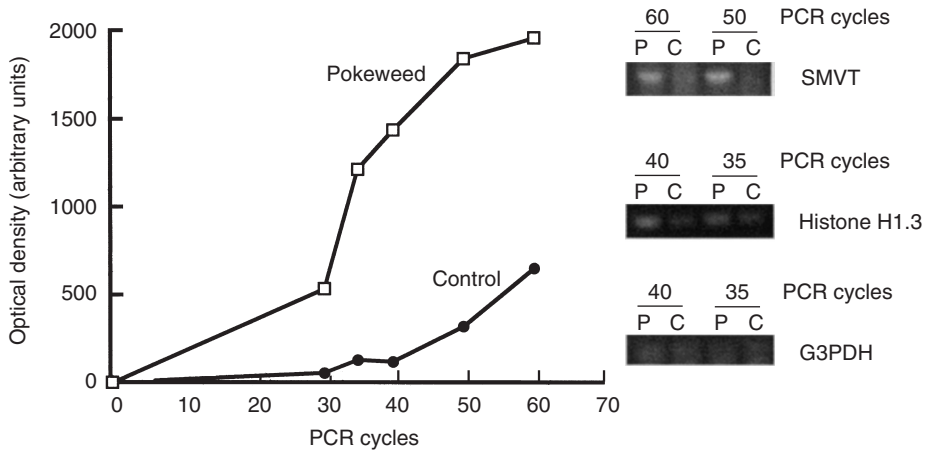

Fig. 12. Expression of the sodium-dependent multivitamin transporter (SMVT) gene in human peripheral blood mononuclear cells. Cells were incubated with 2·0 μg pokeweed (*Phytolacca americana*) lectin (P)/ml for 48 h (□); controls (C) were incubated without mitogen (●). Total RNA was extracted and gene sequences amplified by reverse transcriptase (RT)–polymerase chain reaction (PCR) using primers that were specific for SMVT; histone H1.3 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were quantified by RT–PCR as controls. (From J Zempleni, JS Stanley and DM Mock, unpublished results.)

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