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Synthesis of Tetrahydrobiopterin in Friend Erythroleukemia Cells and Its Modulator Effect on Cell Proliferation

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The induction of the enzymes in the tetrahydrobiopterin pathway by dimethyl sulfoxide (DMSO) was investigated in subclones F4N and B8/3 of the proerythroblastoid Friend erythroleukemia cell line (MEL). GTP-cyclohydrolase, the initial enzyme in the biosynthetic pathway, is virtually absent in both clones, but expression increases during 3 days of DMSO treatment. The final enzyme levels show 12-fold (subclone B8/3) and 40-fold (subclone F4N) increases compared to initial values. Enhancement of 6-pyruvoyl tetrahydropterin synthase activity is detectable 6 h after exposure to DMSO and continues to increase in the 3-day time period to 2.4-fold and 1.8-fold levels in subclones B8/3 and F4N, respectively. Sepiapterin reductase is present in unstimulated F4N cells and absent in B8/3 cells. The enzyme activity is not affected by DMSO treatment in either cell line. This explains why DMSO treatment causes accumulation of tetrahydrobiopterin in the MEL subclone F4N, but not in subclone B8/3. MEL cells are devoid of phenylalanine hydroxylase for which tetrahydrobiopterin serves as cofactor. In F4N, but not in B8/3, tetrahydrobiopterin modulates the rate of [^{3}H]-thymidine incorporation, thus being functionally linked with cell proliferation rather than with differentiation. In contrast to T lymphocytes, periods of tetrahydrobiopterin synthesis and of modulator function are uncoupled in MEL cells.

INTRODUCTION

The *de novo* synthesis of tetrahydrobiopterin (H_4biopterin) begins with GTP (Fig. 1; cf. Ref. [1]). The formation of the first intermediate, dihydroneopterin triphosphate (NH_2TP), is catalyzed by GTP–cyclohydrolase (GTP–CH; EC 3.5.4.16). Its further transformation to H_4biopterin proceeds via tetrahydropterin intermediates and was only recently elucidated [2–5]. Upon triphosphate elimination from NH_2TP, 6-pyruvoyltetrahydropterin synthase (PPH_4 synthase) catalyzes an intramolecular reaction yielding the unstable intermediate, 6-pyruvoyltetrahydropterin (PPH_4). The hydride equivalents for the reduction of this diketo compound to H_4biopterin are provided by NADPH. The enzymes and the sequence of reactions by which they are introduced at positions 1′ and 2′ have not been unequivocally determined. In addition to sepiapterin reductase (SR; EC 1.1.1.153), which potentially catalyzes the reduction of each of the 1′- and 2′-monoketo tetrahydropterins [6], another enzyme, PPH_4 reductase, has been characterized [4, 7]. It was shown to be identical to aldose reductase [8]. Measurement of the contribution of this enzyme in crude cell extracts, however, is not yet possible.

The role of H_4biopterin as a cofactor for the hydroxylation of aromatic amino acids is well established (cf. Ref. [9]). In tissues which are competent for neurotransmitter biosynthesis, changes in the activities of GTP–CH occur in response to physiological conditions and pharmacological treatments [10, 11] and are claimed to be caused by induction of this enzyme [11].

Recent evidence has shown that pterins are also synthesized in tissues and in cell lines lacking aromatic amino acid hydroxylation. In this case, their biosynthesis appears to be related to cell proliferation (cf. Ref. [12]). The formation/synthesis of H_4biopterin during lectin-induced blast transformation [13, 14] is controlled by the activation of both GTP–CH and SR [13]. In primed T-cells and in the $CD4^+$ T-cell lines, HUT 102 and MT 2, activities of GTP–CH, PPH_4 synthase, and SR are controlled by a synergism of both γ-interferon (IFN-γ) and interleukin 2 (IL-2) [14, 16, 17]. The final product, H_4biopterin, in turn, functions as a feedback regulator of T-cell proliferation [18, 19]. It operates by modulation of the IL-2 high-affinity receptor [17, 20].

Data from animal models [21, 22] and from bone marrow transplantations [23–25] suggest that hematopoietic activity correlates with H_4biopterin synthesis. It is terminated during final reticulocyte maturation by a selective loss of GTP–CH activity [26, 27]. The Friend virus-transformed murine erythroleukemia cell line (MEL) is commonly used as a model system for erythropoiesis. It is thought to be arrested before or at the pro-

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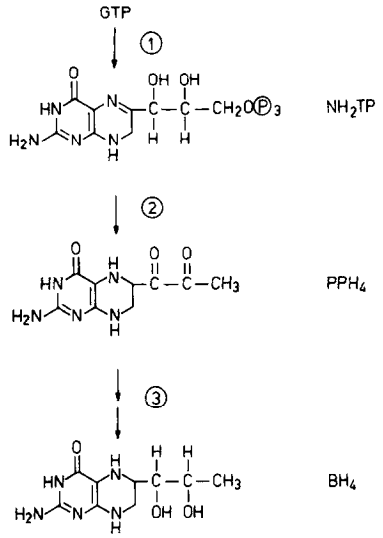

FIG. 1. Biosynthesis of (6R)-5,6,7,8-tetrahydrobiopterin (BH_4). NH_2TP is dihydroneopterin 3′ triphosphate; PPH_4 is 6-pyruvoyltetrahydropterin. (1) GTP-cyclohydrolase I, (2) 6-pyruvoyl-tetrahydropterin synthase, (3) sepiapterin reductase. The involvement of an additional reductase (6-pyruvoyltetrahydropterin (2′-oxo)reductase) has been proposed [4, 7] which was shown to be identical to aldose reductase [8].

erythroblastic stage and possess unrestricted proliferative capacity. The virus-conditioned block of erythropoietic differentiation can be counteracted by a variety of chemically unrelated compounds such as dimethylsulfoxide (DMSO) or hexamethylene bisacetamide (HMBA) (Ref. [28]; cf. Ref. [29]). The result of this treatment mimics many, but not all, of the normal stages of erythropoiesis such as a 10-fold increase in globin mRNA [30] and increased heme production [28]. In addition to the control enzymes for the heme synthesis pathway [31, 32] a range of enzymes such as acetylcholinesterase and carbonic anhydrase are also coordinately induced by erythroid-differentiating chemicals [33]. In other respects the MEL cells undergoing chemically induced differentiation differ from normal erythropoiesis in that their nucleus does not disappear completely [28], glucosephosphate dehydrogenase does not decrease, and catalase activity does not increase [33].

Recent data have shown that MEL subclones may vary considerably with respect to the regulation of H_4biopterin synthesis. In the subclone M18, which does not respond to chemical induction, biopterin levels were unchanged after exposure of the cells to DMSO. In subclone 745 this reagent increases levels two- to threefold [34]. The differentiation-inducing agent HMBA has been reported to cause a decrease in biopterin synthesis in subclone 745 by reducing GTP–CH activity. It has further been demonstrated that stimulation of DNA synthesis was achieved by increasing intracellular H_4biopterin levels [26], suggesting that H_4biopterin also has a functional role in control of cell proliferation in cells of erythroid lineage.

In this study we have analyzed the H_4biopterin-synthesizing system during DMSO treatment of two MEL subclones to address the questions whether it can be induced by this agent and whether the enzymes involved are expressed in a sequential order. We further investigated whether a correlation exists between the formation of H_4biopterin and the expression of aromatic amino acid hydroxylase or whether H_4biopterin also functions as a modulator of S-phase transition in erythroid cells. Two subclones, F4N and B8/3, were examined. In subclone B8/3, in contrast to F4N, an exceptionally rapid accumulation of globin mRNA and a lack of increase in viral RNA during differentiation was found [35]. This indicates major differences between the two subclones and thus further analysis may clarify the conflicting results obtained with different MEL subclones as outlined above.

MATERIALS AND METHODS

Chemicals. All tissue culture reagents were supplied by GIBCO (Bethesda, MD). The origin of the chemicals for sample prepurification and HPLC analysis is listed in Ref. [13]. The Coomassie blue dye reagent was from BioRad (Munich, FRG). DMSO, phenylalanine, tyrosine, dithiothreitol (DTE), trichloroacetic acid (TCA), and all buffer components were from Sigma (St. Louis, MO); GTP and NADPH were from Boehringer-Mannheim (FRG), all pterins were from B. Schircks (Jona, Switzerland), and L-[U-^{14}C]phenylalanine was from Amersham (Braunschweig, FRG). Sephadex G-25 was used as standardized NAP-5 columns from Pharmacia (Freiburg, FRG). The reagents for [^{3}H]thymidine ([^{3}H]TdR) incorporation measurements are listed in Ref. [18].

Cell culture. MEL cells of clones F4N and B8/3 were obtained from W. Ostertag (Universitätsklinik Hamburg-Eppendorf, FRG). The origin of the MEL clones have been described [36]. The cells were maintained in suspension culture with RPMI medium supplemented with 10% heat-inactivated fetal calf serum.

Cells were induced to differentiate by the addition of 1.2% (clone F4N) or 1.7% (clone B8/3) DMSO to logarithmically growing cultures at a density of 1–5 $\times$ 10^5 cells per milliliter. Uninduced control cells were cultured at a density $<1.5 \times 10^6$ cells per milliliter. Cells were harvested by centrifugation, washed once with cold Dulbecco phosphate-buffered saline, and stored at −70°C.

Determination of H_4biopterin. Cellular biopterin was determined after acidic oxidation of the reduced forms by iodine. Deproteinization, prepurification by cation exchange, separation by HPLC, and fluorometric detection of the oxidized pterin have been described previously [13]. The following modifications were used: Pterins were eluted from the cation-exchange resin with 1 *M* NH_4-acetate containing 25% methanol (v/v); monapterin was added as an internal standard prior to the oxidation step.

Enzyme assays. For preparation of cell extracts 2–5 $\times$ 10^7 cells were homogenized in cold 50 m*M* phosphate buffer, pH 7.5. After centrifugation at 12,000*g* for 5 min, the supernatant was desalted on Sephadex G-25 columns equilibrated with the homogenization buffer. GTP–CH, PPH_4 synthase, and SR activities were measured in 50 m*M* phosphate buffer, pH 7.5, at 37°C. GTP–CH activity was determined by the formation of dihydroneopterin triphosphate from GTP. Assays for GTP–CH contained 0.5 m*M* GTP and cell extract in a total volume of 65 μl. After incubation (120 min), the reaction products were oxidized with iodine at pH 1. Neopterin phosphates (NP) were separated by ion-pairing HPLC and monitored by fluorometric detection. The total of neopterin mono-, di-, and triphosphates correspond to enzyme

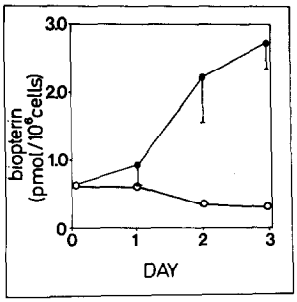

FIG. 2. Increase in H_4biopterin synthesis during DMSO treatment of the MEL subclones B8/3 (○) and F4N (●); ±SD, $n = 6$. The biopterin levels of B8/3 were at the detection limits (0.5–0.6 pmol/10^6 cells) and no SD was calculated.

activity [37]. PPH_4 synthase activity was determined by the formation of tritiated water from [2′-^{3}H]NH_2TP [38]. The specific activity of the substrate was 12.6 GBq mmol^{-1}. The assay mixture contained 0.47 μM [2′-^{3}H]NH_2TP (specific radioactivity 12.6 GBq mmol^{-1}) and 8 mM $MgCl_2$ and protein in a total volume of 30 μl. They were incubated at 37°C for 120 min. Tritiated water was measured as described [38].

SR activity was determined by the formation of H_2biopterin from sepiapterin. The assay contained 40 μM sepiapterin, 100 μM NADPH, and cell extract in a total volume of 300 μl. The reaction product, H_2biopterin, was determined after acidic iodine oxidation to biopterin by reversed-phase HPLC as described [13].

Phenylalanine hydroxylase (PAH) was measured by conversion of L-[U-^{14}C]phenylalanine to [^{14}C]tyrosine [39]. Tyrosine was separated from phenylalanine by HPLC using the ion-pair solvent system described above. The amino acids were identified by the UV spectra of the coeluting unlabeled species by means of the Hewlett–Packard HP 1040A detection system. The assay system (100 μl final volume) contained 0.1 M phosphate buffer, pH 6.8, L-[U-^{14}C]phenylalanine (final concentration 0.4 mM), and DTE (final concentration 10 mM) [40]. The assay was run for 5 min (mouse liver) or 60 min (MEL cells) at 27°C. It was stopped by the addition of 20 μl 2 M TCA. The radioactivity of the tyrosine and phenylalanine fractions was determined by a Beckman scintillation spectrometer. PAH activity was calculated from the percentage conversion of [^{14}C]phenylalanine to tyrosin and the substrate concentration. Protein concentrations for all assays were determined by the Coomassie blue dye assay with bovine serum albumin as standard.

[^{3}H]TdR incorporation. Cells were harvested by centrifugation from an exponentially growing culture and were seeded in 200-μl microtiter wells at a density of 3–6 × 10^5/ml. H_4biopterin and sepiapterin were added from freshly prepared stock solutions and were serially diluted with culture medium. 2-Mercaptoethanol which had been added to the stock solutions resulted in a final concentration of 10.5 nM in the first well. It had no apparent effect on the [^{3}H]thymidine incorporation rate. At the periods indicated, cells were pulse-labeled for 1 h and then semiautomatically harvested as described in Ref. [18].

RESULTS

Synthesis of Cellular Biopterin during DMSO Induction

The concentration of H_4biopterin during DMSO induction was studied in subclones F4N and B8/3 of the MEL cell line. Only trace amounts of biopterin (0.6–0.7 pmol/10^6 cells) were found in both subclones. In each of six experiments DMSO treatment of F4N cells caused a marked increase in cellular biopterin levels which started after 24 h and reached maximum values on Day 3 (Fig. 2). In contrast, biopterin did not increase in B8/3 cells upon DMSO treatment.

The Biopterin-Synthesizing Enzymes during DMSO Induction

Neopterin production by GTP–CH was close to the detection limits (0.1 pmol min^{-1} mg^{-1}) in both F4N and B8/3 subclones. In each of five experiments DMSO treatment caused a 40-fold increase in GTP–CH activity in F4N cells during a period of 3 days (Fig. 3). The same time course was followed in B8/3 cells, but the final activity was increased only 12-fold compared to that of controls.

The development of PPH_4 synthase activity upon DMSO treatment was determined at nonsaturating substrate conditions for two reasons. First, maximum sensitivity was achieved when the substrate was used at the

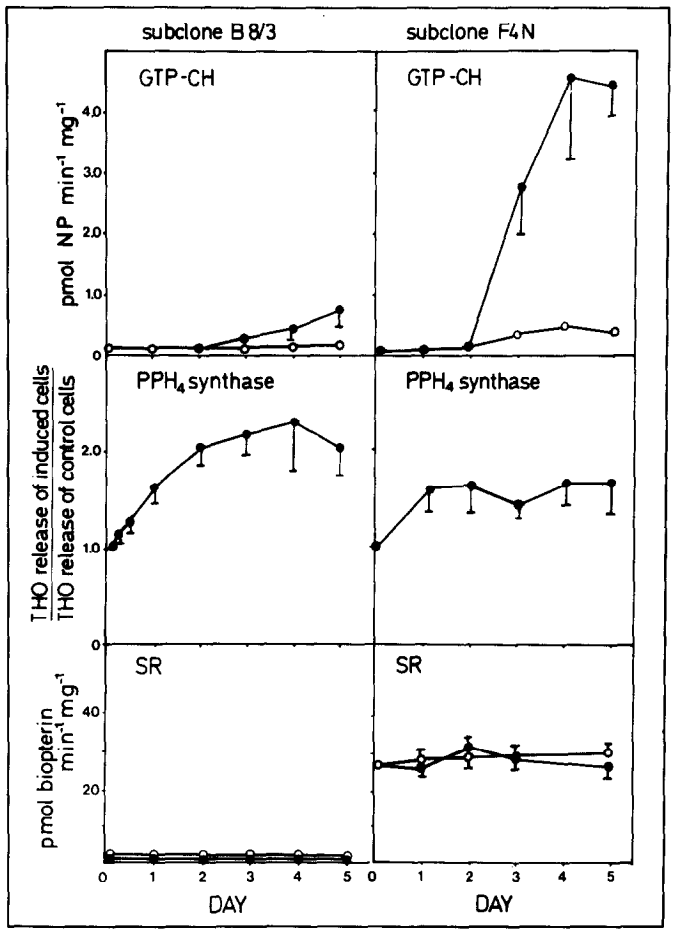

FIG. 3. Induction of GTP–CH, PPH_4 synthase, and SR activities during DMSO treatment of the MEL subclones B8/3 (left lane) and F4N (right lane). (○) Control cells; (●) addition of 1.7% (clone B8/3) and 1.2% (clone F4N) DMSO to the medium; ±SD, $n = 5$. For values at the detection limits (GTP–CH: 0.1–0.2 pmol mg^{-1} min^{-1}; PPH_4 synthase 30 fmol mg^{-1} min^{-1}; SR: 2 pmol mg^{-1} min^{-1}) no SD was calculated. NP, total of neopterin mono-, di-, and triphosphate.

TABLE 1

K_m Values for PPH_4 Synthase and its Specific Activities at Substrate Saturating Conditions in Control MEL Cells

	K_m ($\times 10^{-6} M$)	V_{max} (pmol min^{-1} mg^{-1})
Subclone B8/3	2.42	19.7 ± 4.4 (n = 5)
Subclone F4N	2.77	17.0 ± 5.9 (n = 5)

highest activity available (see Methods). Second, our studies focused on relative changes during differentiation. To compensate for variations in the individual induction experiments, DMSO-induced changes in PPH_4 synthase activities are expressed relative to control cells (Fig. 3). The graph demonstrates that in each of five experiments DMSO induced an immediate increase in activity which was detectable after 6 h and continued for 3 days in subclone B8/3, resulting in 2.5-fold final levels. PPH_4 synthase in F4N cells reached about 1.8-fold levels after Day 1 and showed no further increase.

PPH_4 synthase follows normal Michaelis-Menten kinetics [38]. The apparent K_m values were determined by standard curves using lower specific radioactivities and thus V_{max} under substrate-saturating conditions could also be calculated for the enzyme from MEL cells (Table 1). This allows comparison of its activity in control cells with both of the other biosynthetic enzymes which are presented in this study in a conventional manner for substrate-saturating conditions (t_0 in Fig. 3). The values demonstrate that GTP–CH, rather than PPH_4 synthase or SR is the rate-limiting enzyme in control and also in DMSO-induced F4N cells.

In F4N cells SR activities remained unchanged after DMSO treatment (Fig. 3, right lane). On the contrary, no SR was found in subclone B8/3 and the block in each of five experiments could not be counteracted by DMSO treatment. This explains the finding that H_4biopterin cannot develop upon DMSO induction in these cells.

Absence of Phenylalanine Hydroxylase (PAH) in MEL

To address the question whether H_4biopterin synthesis in MEL cells is related to its known cofactor function, we have examined whether PAH activity is constitutively present in MEL or can be induced by DMSO. The activity of PAH in mouse liver extract was found to be 2.43 ± 0.38 nmol tyrosine min^{-1} mg^{-1} (n = 4). No activity could be detected in uninduced or in DMSO-induced MEL cells from either subclone.

Modulation of [^{3}H]TdR Incorporation Rate by H_4biopterin

The apparent lack of cofactor function prompted us to examine whether H_4biopterin modulates the proliferation of MEL cells similarly as in T-cells [18, 19]. H_4biopterin and sepiapterin added to the cells at the time of seeding significantly increased the rate of DNA synthesis in the rapidly dividing cells of subclone F4N. They were inactive in cells from subclone B8/3. Maximum enhancement was found in the range 5–9 $\times 10^{-7}$ M (Fig. 4). No toxic effect on either subclone could be observed at these pterin concentrations.

In F4N dihydrofolate reductase activity amounts to 1.19 ± 0.41 nmol NADPH mg^{-1} min^{-1} and high activities of SR were also found (Fig. 3). Thus, by sequential activity of SR and DHFR these cells readily convert sepiapterin to H_4bipterin via the salvage pathway [41]. Indeed, intracellular H_4biopterin levels increase to 60 pmol/10^6 cells after incubation with sepiapterin for 3–8 h. It can therefore be concluded that sepiapterin becomes effective after conversion to H_4biopterin. This pathway has also been suggested for the sepiapterin-mediated increase in proliferation in subclone 745 [26]. In subclone B8/3 the activity of DHFR was the same (1.21 ± 0.21 nmol NADPH mg^{-1} min^{-1}) as in F4N, but due to the lack of SR activity (Fig. 3) no conversion to H_4biopterin was found. The failure of these cells to respond immediately to H_4biopterin, however, suggests that the DNA synthesis rate in B8/3, contrary to that in F4N, is not modulated by the final product of the biosynthetic pathway.

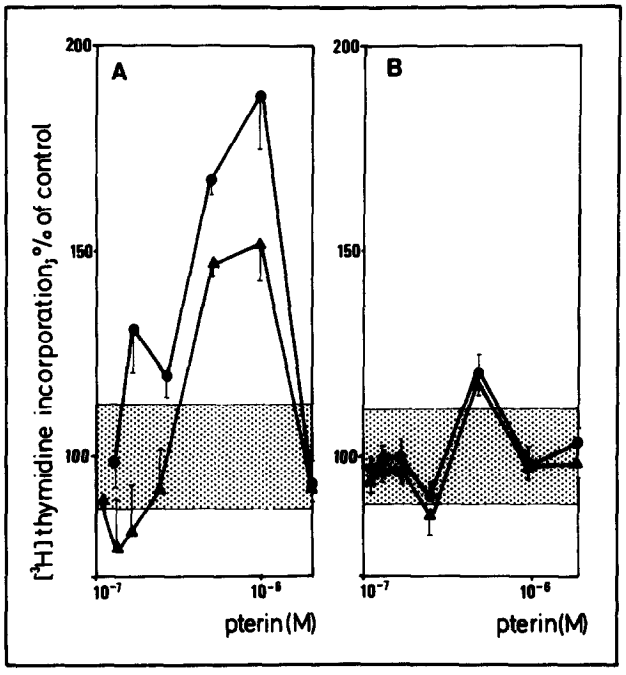

FIG. 4. Effect of H_4biopterin and sepiapterin on DNA synthesis in MEL subclones F4N (A) and B8/3 (B). Cells from exponentially growing cultures were seeded with pterins at the concentrations indicated. [^{3}H]TdR incorporation was measured after 19 h by pulse labeling for 1 h. (●) H_4biopterin; (▲) sepiapterin. Dotted area: 95% probability levels of DNA synthesis in medium alone.

DISCUSSION

A first point to emerge from the present study is that H_4biopterin synthesis in both F4N and B8/3 subclones of MEL cells is blocked. The enzymes of the *de novo* H_4biopterin synthesis are individually and selectively affected. GTP–CH and PPH_4 synthase are initially absent, but are induced by DMSO treatment in both subclones with similar time courses, even though the final levels are different. SR appears to be governed by a different program since it is not subject to the block in subclone F4N, but is irreversibly suppressed in subclone B8/3. Therefore, DMSO treatment does not commit these MEL cells to H_4biopterin synthesis. B8/3 is the first cell type found to lack SR activity, so far.

In both subclones the induction of PPH_4 synthase precedes the induction of GTP–CH, although the latter catalyzes the first step in the biosynthetic pathway. Noncoordinate expression has also been reported for other pathways, e.g., for commitment to terminal cell division [42]. It should be noted that the accumulation of H_4biopterin in F4N cells precedes the induction of GTP–CH by 24 h. A similar divergence in these time courses was induced in the HTLV-I-transformed T cell line MT-2 by synergistic action of IFN-γ and IL-2 [17]. A "salvage pathway" has been postulated to exist in addition to *de novo* biosynthesis in a number of cell lines. It proceeds from intermediates which have not yet been characterized [41]. Whether this pathway also operates in MEL cells and thus gives rise to the early increase in H_4biopterin levels after DMSO treatment remains to be further elucidated.

In our study F4N responds to DMSO in a way similar to that which has been reported for subclone 745 [34]; namely, biopterin production increases upon treatment with this agent. In contrast, HMBA was found to terminate H_4biopterin synthesis in the same subclone through a decrease in GTP–CH activity [26]. Treatment with either agent, however, induces differentiation as indicated by hemoglobin synthesis [26, 34]. The conflicting data suggest that H_4biopterin synthesis is not coupled to the events resulting in differentiation. Second, they demonstrate that MEL subclones may react individually and differently, imposing limitations for a generalized use of MEL cells as a model system for studying erythropoiesis.

Since MEL cells express PAH activity neither by constitution nor after induction with DMSO, a functional role for endogenous H_4biopterin in the biosynthesis of tyrosine can clearly be ruled out. An additional role of H_4biopterin synthesis with respect to the control of cell proliferation has been documented for the MEL subclone 745 [26]. The present study demonstrates a similar phenomenon in subclone F4N, but not in subclone B8/3. As in activated T-cells [18, 19], the enhancement of [^{3}H]TdR incorporation in F4N cells follows an optimum curve with respect to H_4biopterin concentration. In both cases it culminates at $6–9 \times 10^{-7}$ *M*. In the case of clonal expansion of T-cells, H_4biopterin appears to operate via modulation of IL-2 receptor assembly [17, 20]. The pathway of its operation in MEL cells has not been identified.

It should be pointed out that in T-cells H_4biopterin synthesis and its modulator function are temporally coordinated. Periods of H_4biopterin synthesis closely precede the periods of main DNA synthesis during blast transformation of resting T-cells [13, 14] and during IL-2-induced proliferation of primed T-cells [16]. In spite of the fact that MEL subclone F4N responds to exogenous H_4biopterin, the events are uncoupled in these cells. In subclone B8/3, on the other hand, both H_4biopterin synthesis and responsiveness are lost.

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