

Tetrahydro-6-biopterin is associated with tetrahydro-7-biopterin in primary murine mast cells

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Murine bone marrow-derived mast cells proliferate in response to interleukin 3. In addition to 6-biopterin, 7-biopterin was identified in these cells by HPLC analysis of iodine oxidized extracts and by alkaline permanganate oxidation to the 6- and 7-carboxylic acids. 7-Biopterin comprised 31.9 (± 7.7)% of the total biopterin. It was absent in cells which were grown with of L-*p*-chlorophenylalanine, an inhibitor of tryptophan 5-mono-oxygenase. Both 6- and 7-biopterin were present in the cell as their tetrahydro forms. From these data we conclude that 7-biopterin, in contrast to e.g. brain tissue, regularly occurs as a normal metabolite in primary mast cells and that it is generated during hydroxylation of tryptophan.

Mast cell; Tetrahydro-6-biopterin; Tetrahydro-7-biopterin; 6-Biopterin; 7-Biopterin; Tryptophan 5-mono-oxygenase

1. INTRODUCTION

The *de novo* biosynthesis of H₄-6-biopterin begins with GTP. The first step is catalyzed by GTP-cyclohydrolase I (EC 3.5.4.16) and results in the formation of dihydro-6-neopterin triphosphate. This intermediate is transformed into H₄-6-biopterin by the sequential action of 6-pyruvoyl-H₄pterin-synthase and sepiapterin reductase (EC 1.1.1.153) (cf. [1]). H₄-6-biopterin is the natural and immediate electron donor for the hydroxylation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan thus controlling the biosynthesis of the neurotransmitters epinephrine, dopamine, and serotonin as well as the catabolism of phenylalanine (for review see [2]). In the course of the hydroxylation reaction, H₄-6-biopterin is oxidized to the 4a-hydroxy H₄biopterin (4a-carbinolamine) intermediate. Rapid formation of quinonoid dihydro-6-biopterin is achieved by the catalytic action of 4a-carbinolamine dehydratase [3,4]. It is consensus view that the mechanisms of all of the aromatic amino acid hydroxylases are the same and

proceed via the 4a-carbinolamine intermediate of the reduced pteridine (cf. [2,5]).

7-Biopterin was found to contribute 3.1 and 2.7% to the total biopterin found in human and mouse liver, respectively. Only trace amounts of this isomer were found in the urine of normal individuals [6]. In patients with a new variant of H₄-6-biopterin deficiency and evident hyperphenylalaninemia the urinary excretion of 7-biopterin was increased and in some cases approached a 1:1 ratio as compared with 6-biopterin [6]. It had been strongly suggested [7] and then finally demonstrated [8] that the *in vitro* formation of 7-biopterin occurs via a non-enzymatic re-arrangement of 4a-hydroxy H₄biopterin which results from an opening of the pyrazine ring and its recyclization. A genetic defect is assumed to cause increased 7-biopterin levels in patients with hyperphenylalaninemia [7].

Recent evidence has shown that H₄-6-biopterin is synthesized during multiplication and differentiation of cells lacking neurotransmitter biosynthesis and phenylalanine degradation. This is the case during hematopoiesis and cellular immune responses (cf. [9]). Interferon- γ , in cooperation with interleukin 2, was identified as the factor which triggers neopterin and H₄-6-biopterin synthesis in cells of the immune system [10,11]. To further address H₄-6-biopterin formation during cytokine-directed cell proliferation, bone marrow-derived murine mast cells were analyzed. The multiplication of these primary mast cells is directed by interleukin 3 [12] and they produce serotonin [13]. In this study we demonstrate that in contrast to brain tissue, H₄-6-biopterin from the primary mast cells is accompanied by substantial amounts of the 7-isomer.

Abbreviations and trivial names: H₄-6- or 7-biopterin, 6- or 7-(L-erythro-1',2'-dihydroxy-propyl)-5,6,7,8-tetrahydropterin; 6- or 7-biopterin, 6- or 7-(L-erythro-1',2'-dihydroxypropyl)-pterin; 6- or 7-neopterin, 6- or 7-(L-erythro-1',2',3'-trihydroxypropyl)-pterin; monapterin, 6-(L-threo-1',2',3'-trihydroxypropyl)-pterin; 6-pyruvoyl-H₄pterin, 6-pyruvoyl-5,6,7,8-tetrahydropterin; IL-3, interleukin 3.

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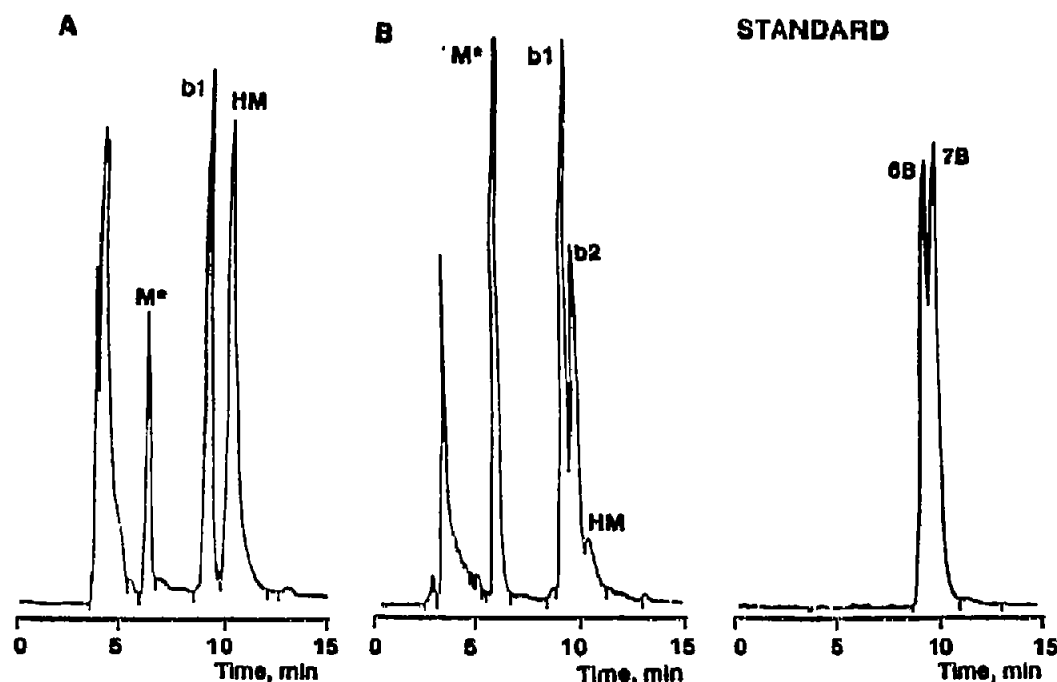


Fig. 1. Reverse-phase HPLC analysis of brain tissue and of mast cells extracts after acidic iodine oxidation (solvent I). A, murine brain tissue; B, murine bone marrow derived mast cells. M* Monapterin which was added as standard prior to the oxidation step; b1, identified with 6-biopterin; b2, identified with 7-biopterin; HM, 6-hydroxy-methyl-pterin.

2. MATERIALS AND METHODS

2.1. Materials

The following materials were obtained from the manufacturers in parentheses: Murine (m) recombinant (r) IL-3 (Bachem, Heidelberg), cell culture components (Biochrom, Berlin), L-p-chlorophenylalanine (Sigma, München), HPLC columns (Bischoff, Leonberg). 7-Biopterin was a gift from Dr. M. Viscontini (Zürich); all other pteridines were from Schireks (Jona, Switzerland).

2.2. Cell culture

Homogenous populations of mast cells (>99% Alcian blue-positive) were obtained from bone marrow cultures of normal BALB/c mice as described previously [14]. Primary mast cells (in vitro age: 3 to 4 weeks) were cultured at 5×10^5 cells ml^{-1} in RPMI 1640 medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, 10^{-5} M alpha-thioglycerol, 100 U ml^{-1} penicillin-streptomycin, and 10 ng ml^{-1} mIL-3. After an incubation period of 40 h the mast cells were harvested and stored at -70°C . L-p-chlorophenylalanine (5 mM) was added at t_0 of this incubation period, when indicated.

2.3. Pteridine analysis

Extracts from mast cells ($1-2 \times 10^6$) were prepared with 1 ml 1 N HCl; extracts from whole murine brain were prepared with 10 ml 1 N HCl and 2 ml aliquots were processed further. 6-biopterin was oxidized by acidic iodine, deproteinized and pre-purified by cation-exchange as previously described [15]. Modifications of the method have been detailed in [16]. For the quantification of dihydrobiopterin aliquots of the acidic extracts were brought to pH 13. Oxidation with iodine (in 0.1 M NaOH) was followed by addition of 2 N HCl to change biopterin back into its cationic form [17]. The pre-column and the separation column (250 $\times$ 4.6 mm) of the HPLC system consisted of Shandon Hypersil-ODS 5 μm . A Shimadzu model RF S35 HPLC fluorescence detector was interfaced with a Shimadzu C-R6A Chromatopac analytical data system. The following pterins were used for

reference: 6-biopterin, 7-biopterin; 6-neopterin, 7-neopterin, monapterin, D-threo-biopterin, 6-hydroxymethylpterin, 2-amino-4-oxo-pteridine, pterin-6-carboxaldehyde, pterin-6-carboxylic acid, and pterin-7-carboxylic acid. The following solvents were used: (I) 3% methanol, 1% acetonitrile, pH 2.5 was adjusted with H_3PO_4 ; (II) 0.5% isopropanol, 0.5% methanol, pH 5.2 was adjusted with acetic acid; (III) sodium acetate 50 mM, citric acid 5 mM, pH 5.2 was adjusted with NaOH; (IV) 5% methanol, sodium phosphate 100 mM, pH 3 was adjusted by H_3PO_4 . The flow rate was 1 $\text{ml} \cdot \text{min}^{-1}$ at room temperature (20°C). For oxidation of pterins to the corresponding carboxylic acids (cf. [18]) fractions 'b1' and 'b2' were purified by HPLC in solvent I, concentrated by lyophilization and subjected to the alkaline permanganate method as described in [8].

2.4. Determination of tryptophan 5-mono-oxygenase

Extracts from $2-3 \times 10^7$ mast cells were prepared and desalted as described in [11]. The enzyme was activated and then assayed as described in [19]. The reaction product 5-hydroxytryptophan was separated by reverse phase HPLC (mobile phase 0.01 M sodium acetate containing 15% methanol brought to pH 4.85 with acetic acid) and determined by its fluorescence (excitation 285 nm, emission 345 nm).

3. RESULTS AND DISCUSSION

The HPLC chromatograms of iodine-oxidized extracts from murine brain tissue separated two pteridines, 'b1' and a slower migrating fraction (Fig. 1A). Comparison with the standard pterin mixture (see section 2) in solvents I, II, and III identified the latter as 6-hydroxymethylpterin (data not shown). In contrast to brain tissue, HPLC chromatograms from primary murine mast cells with solvent (I) (Fig. 1B) and II (data not

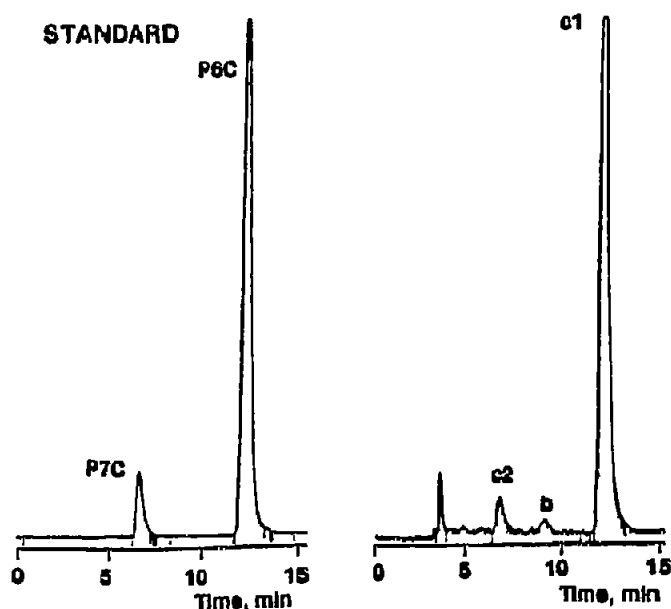


Fig. 2. Reverse-phase HPLC of the pterin products formed after alkaline permanganate oxidation of 'b1' and 'b2' in solvent IV. c1, identified with 6-carboxylic-acid (P6C); c2, identified with 7-carboxylic-acid (P7C); b, non-oxidized 6- and 7-biopterin (<0.1% of total). 7-Carboxylic-acid shows 9% of the relative fluorescence intensity as compared to the 6-isomer. Fluorescent fractions close to the void volume were also found in lyophilized and mock oxidized solvent I.

shown) separated two major pterin fractions 'b1' and 'b2'. 6-Hydroxymethylpterin was either absent or comprised <10% of total pterins.

The identification of 'b1' and 'b2' is addressed in more detail below. Advantage was taken of the fact that 6-biopterin separates from 7-biopterin in solvents I and II (Fig. 1, standard). As expected, a comparison of HPLC chromatograms with the reference pterins showed that fraction 'b1' from brain tissue [17] and from the mast cells was identical with 6-biopterin. Fraction 'b2' which was only found in mast cells was identical with 7-biopterin. It co-eluted with this isomer in solvent I (Fig. 1B) and II (data not shown) which separate 6- from 7-biopterin. Its retention time was clearly different from the other pterins in at least one of the solvent systems I, II, or III. Alkaline iodine oxidation of the cell extract destroys H_4 -biopterin, but oxidizes H_2 -biopterin to biopterin [17]. After this treatment neither 6- nor 7-biopterin was detectable in the extracts of both mast cells and brain tissue. Therefore, it can be concluded that both fractions 'b1' and 'b2' are present as tetrahydro derivatives.

Alkaline permanganate treatment of 6- and 7-substituted pterins oxidizes the side chains to the corresponding carboxylic acids (cf. [18]) which were separated in HPLC buffer IV [8] (Fig. 2). Pterin 7-carboxylic acid is characterized by a marked reduction of its relative fluorescence intensity at 450 nm as compared to the 6-isomer [8]. In our experiments, it displayed only 9% of

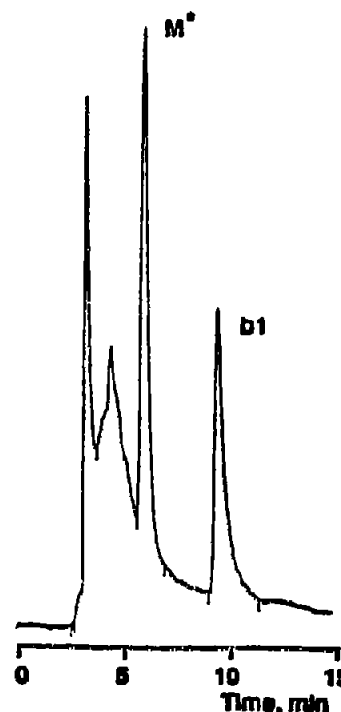


Fig. 3. Reverse-phase HPLC analysis of mast cell extracts after acidic iodine oxidation (solvent I). The cells were grown for 40 h with *l*-*p*-chlorophenylalanine (5 mM) before harvest. For explanation of symbols see Fig. 1.

that produced by pterin-6-carboxylic acid. HPLC separation of pterin 6- and 7-carboxylic acid (Fig. 2) and of the oxidation products obtained from the putative 6- and 7-biopterins of mast cells (Fig. 2) yielded identical elution profiles. Almost the same portion of total pterin carboxylic acid was recovered in form of the 7-isomer after permanganate treatment (30.5%) as previously had been present as putative 7-biopterin (32.3%).

A greenish, instead of a blue fluorescence has been reported for pterin-7-carboxylic acid after separation by paper chromatography with 100 mM KH_2PO_4 [20]. It lacks a discrete fluorescence emission maximum at 450 nm as is found for pterin-6-carboxylic acid but is characterized by an extended fluorescence emission band including longer wave lengths (H. Rembold, personal communication). This property was used as a further criterion for identifying the mast cell derived oxidation products as pterin-6- and 7-carboxylic acid. A change in the settings for fluorescence excitation from 350 to 360 nm and for fluorescence emission from 450 to 475 nm reduced the signal intensity of both the synthetic and the putative mast cell-derived pterin-7-carboxylic acid to only 34% and 36%, respectively, whereas the signal of pterin-6-carboxylic acid from both origins was reduced to only 95% and 85%, respectively. From these data we conclude that in primary mast cells H_4 -6-biopterin is associated with its 7-isomer whereas in brain tissue only the 6-isomer is found.

Table I

6-Biopterin and 7-biopterin in murine interleukin 3-responsive mast cells. Primary mast cells derived from 4 different mouse bone marrow specimens were cultured in the presence of mIL-3 (10 ng ml^{-1}) for 40 h. The tetrahydropterins were determined after acidic iodine oxidation of the cell extract

Primary mast cells	6-biopterin pmol/ 10^6 cells	7-biopterin pmol/ 10^6 cells	%7-isomer of total biopterin
LD63	4.2	2.9	40.8
LD70	2.5	0.8	24.2
LD75	5.6	3.1	35.6
LD76	4.6	1.7	27.0
$\bar{x} \pm \text{S.D.}$	4.2 ± 1.3	2.1 ± 1.1	31.9 ± 7.7

Table I shows that H_4 -7-biopterin averages $31.9 (\pm 7.7)\%$ of the total H_4 -biopterin in the mast cells. This demonstrates that substantial amounts of this isomer do originate not only under in vitro phenylalanine hydroxylase assay conditions by omission of 4a-dehydratase [8] or in patients suffering from hyperphenylalaninemia who are supposed to be defective in this enzyme [6,7]. The data suggests that it rather originates also under in vivo conditions in primary mast cells during the course of the tryptophan 5-mono-oxygenase (EC 1.14.16.4) reaction which shares the same mechanism with the other aromatic amino acid hydroxylases.

The activity of tryptophan 5-mono-oxygenase in the primary mast cells was $620 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein before activation and $980 \text{ pmol min}^{-1} \text{ mg}^{-1}$ protein after activation by DTT and Fe^{2+} . *p*-Chlorophenylalanine inhibits phenylalanine 4-mono-oxygenase (EC 1.14.16.1) and tryptophan 5-mono-oxygenase under in vitro as well as under in vivo conditions [19,21]. Cultivation of the mast cells with this inhibitor caused absence of 7-biopterin (Fig. 3). 6-Biopterin was identified by co-elution with the standard (see Fig. 1). Its level increased to $6.7 (\pm 0.4) \text{ pmol/}10^6$ cells, thus compensating for the 7-isomer. It is therefore to be concluded that the 7-isomer in primary mast cells originates from the tryptophan 5-mono-oxygenase reaction.

The absence of 7-biopterin in brain tissue, however, remains an open question. It is well documented that tryptophan 5-mono-oxygenase from a non-neuronal source such as the murine mast cell line P815 [19,23] or from intestinal mucosa [24] has different functional and regulatory properties. The non-neuronal enzyme needs to be activated and stabilized by DTT and Fe^{2+} prior to the assay [19,24]. Most likely, the enzymes from both sources represent distinct molecular entities [5]. The absence of 7-biopterin in brain tissue raises the question whether neuronal and non-neuronal tryptophan 5-mono-oxygenases differently associate with 4a-dehydratase, thus affecting the recyclozation of the H_4 -6-

biopterin cofactor or whether the activity of the dehydratase in non-neuronal tissues is low.

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