Synthesis and characterization of ^{3}H-labelled tetrahydrobiopterin

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We synthesized [3′-^{3}H]-5,6,7,8-tetrahydrobiopterin from [8,5′-^{3}H]guanosine 5′-triphosphate ([8,5′-^{3}H]GTP) using GTP cyclohydrolase I (EC 3.5.4.16), 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase (EC 1.1.1.153). After purification by cation-exchange h.p.l.c. a solution of radiochemically pure (> 95%) [3′-^{3}H]-5,6,7,8-tetrahydrobiopterin with a specific activity of 9.2 Ci/mmol was obtained. The product proved well suited for studying the binding of tetrahydrobiopterin to nitric-oxide synthase.

INTRODUCTION

5,6,7,8-Tetrahydrobiopterin is formed in mammals from GTP. Thus far, five oxygenase reactions are known to be promoted by tetrahydrobiopterin: phenylalanine 4-mono-oxygenase (EC 1.14.16.1), tyrosine-3-mono-oxygenase (EC 1.14.16.2), tryptophan 5-mono-oxygenase (EC 1.14.16.4), glyceryl-ether mono-oxygenase (EC 1.14.16.5) and nitric-oxide synthase (EC 1.14.13.39, reviewed in [1]). In phenylalanine mono-oxygenase, tetrahydrobiopterin is oxidized stoichiometrically with product formation, the oxidized 6,7(8*H*)-dihydrobiopterin cofactor diffuses from the enzyme and is reduced back to the active tetrahydro compound by dihydropteridine reductase [1]. The role of tetrahydrobiopterin in nitric-oxide synthase, however, is still a matter of debate, since tetrahydrobiopterin stimulates nitric-oxide synthase turnover in substoichiometric amounts [2] and is not recycled outside the nitric-oxide synthase [3]. On the other hand it has been shown that intracellular concentrations of tetrahydrobiopterin, which are increased by the action of cytokines (reviewed in [4]), can limit the activity of nitric-oxide synthase in some cells [5]. Some tetrahydrobiopterin remains bound to nitric-oxide synthase upon purification [2] and promotes assembly of the active dimeric structure of the enzyme [6]. To get a new means of assessing the pteridine binding-site of nitric-oxide synthase, we synthesized 6*R*-[3′-^{3}H]-5,6,7,8-tetrahydrobiopterin from [8,5′-^{3}H]GTP by using GTP cyclohydrolase I, 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase. We compare this procedure with chemical synthesis of [6-^{3}H]-5,6,7,8-tetrahydrobiopterin from 7,8-dihydrobiopterin by reaction with [^{3}H]$NaBH_4$ [7]. Finally, we demonstrate the use of 6*R*-[3′-^{3}H]-5,6,7,8-tetrahydrobiopterin for examining the binding of tetrahydrobiopterin to nitric-oxide synthase purified from porcine brain.

MATERIALS AND METHODS

Reagents

[8,5′-^{3}H]GTP (33 Ci/mmol) and [^{3}H]$NaBH_4$ (54 Ci/mmol) were obtained from New England Nuclear (Vienna, Austria), pteridines were from Schircks Laboratories (Jona, Switzerland), NADPH, isopropyl-β-D-1-thiogalactoside and protamine sulphate were from Serva (Heidelberg, Germany), lysozyme, DNAse I, dihydropteridine reductase, ampicillin, kanamycin and 2,6-dichlorophenol-indophenol were from Sigma (Deisenhofen, Germany), and LB medium was from Difco (Detroit, MI, U.S.A.). All other chemicals were the highest-purity grade available from Merck (Darmstadt, Germany). Protein concentrations were determined by the method of Bradford [8], using pure BSA (Serva) as standard and the dye reagent from Bio-Rad (Vienna, Austria).

Chromatographic instruments and columns

F.p.l.c. was performed on a 1050 titanium h.p.l.c. from Hewlett-Packard (Vienna, Austria) equipped with columns: Fractogel TSK 650 Butyl, Fractogel TSK 650 DEAE (both 10 mm inner diameter, 150 mm length, 25–40 μm particle size, Merck), Superose 12 (10 mm inner diameter, 300 mm length, 30 μm particle size, Pharmacia, Uppsala, Sweden) and a 10 ml Superloop for sample application (Pharmacia). H.p.l.c. was performed with a stainless steel 1050 h.p.l.c. (Hewlett-Packard) and columns: Nucleosil 10 SA (4 mm inner diameter, 250 mm length, 10 μm particle size, Marcherey Nagel, Düren, Germany) and Lichrospher RP-18 (4 mm inner diameter, 25 mm or 250 mm length, 5 μm particle size, Merck). Fractions were collected with an L-5200 collector from Merck.

Enzymes

GTP cyclohydrolase I was prepared from *Escherichia coli* strain M15 carrying a plasmid encoding for *E. coli* GTP cyclohydrolase I under control of the *lac* repressor (kindly donated by A. Bacher, Technische Universität München, Germany). Bacteria were grown in 1 litre of LB medium, containing ampicillin (150 mg/l) and kanamycin (25 mg/l) to an absorbance (600 nm) of 0.6–0.8 and treated with 2 mM isopropylthiogalactoside for 20 h. Bacteria were collected by centrifugation (13000 *g*, 20 min, 4 °C), lysed by incubation at 37 °C for 1.5 h in 4 ml of Buffer A [10 mM potassium phosphate/2.5 mM EDTA/0.02% (w/v) NaN_3, pH 7.0], containing lysozyme (0.25 mg/ml) and DNAse I (0.025 mg/ml). The enzyme was purified by f.p.l.c. on DEAE at a flow rate of 1 ml/min using elution for 20 min with 100% buffer A followed by a linear gradient to 100% buffer B (buffer A + 1 M KCl) for an additional 75 min. The enzyme eluted at 400 mM KCl; the activity was determined by assessing the amount of 7,8-dihydroneopterin triphosphate formed from GTP as follows. A 5 ml portion of the fraction was incubated in 100 μl 0.1 M Tris/2.5 mM EDTA/0.3 M KCl/1 mM GTP (freshly

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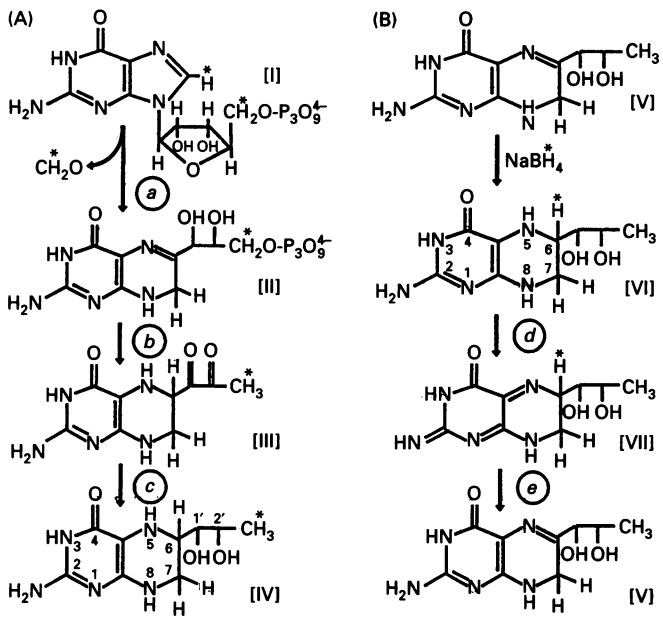

Scheme 1 Strategies of enzymic (A) and chemical (B) labelling of tetrahydrobiopterin with ^3H and loss of the 6-^3H-label upon oxidation

Enzymic (A) and chemical (B) labelling as well as oxidation of [6-^3H]-5,6,7,8-tetrahydrobiopterin was carried out as detailed in the Materials and methods section. Notation of compounds: [I], [8,5′-^3H]GTP; [II], [3′-^3H]-7,8-dihydroneopterin triphosphate; [III], 6*R*-[3′-^3H]-6-pyruvoyl-5,6,7,8-tetrahydropterin; [IV], 6*R*-[3′-^3H]-5,6,7,8-tetrahydrobiopterin; [V], 7,8-dihydrobiopterin; [VI], 6-*R*,*S*-[6-^3H]-5,6,7,8-tetrahydrobiopterin; [VII], [6-^3H]-6,7(8*H*)-dihydrobiopterin. *a*, GTP cyclohydrolase I; *b*, 6-pyruvoyltetrahydropterin synthase; *c*, sepiapterin reductase; *d*, chemical or enzymic oxidation; *e*, spontaneous rearrangement.

added)/10% (v/v) glycerol for 15 min at 37 °C; 5 μl of HCl (1 M) and 5 μl of I_2 (0.1 M in 0.25 M KI) were added to terminate the reaction and oxidize the 7,8-dihydro derivative to the fluorescent neopterin triphosphate. After incubation for 15 min in the dark, 10 μl of 0.1 M ascorbic acid was added to destroy excessive iodine. Finally, the neopterin phosphates were quantified by h.p.l.c. as follows. A 10 μl volume was injected on to a 25 mm RP-18 column equilibrated with 15 mM potassium phosphate buffer, pH 6.4 at a flow rate of 0.8 ml/min. The neopterin phosphates were detected by fluorescence using an excitation wavelength of 350 nm and an emission wavelength of 440 nm. Calibration of the amount of the neopterin phosphates was performed by comparison of the area of the fluorescent peaks to the area resulting from synthetic neopterin standards. Detailed investigations showed that this shortened protocol yields results comparable with the method usually employed [9] which measures neopterin after cleavage of the phosphate groups by alkaline phosphatase. The GTP cyclohydrolase I preparations obtained had specific activities of 320–370 nmol·min^{-1}·mg^{-1}.

6-Pyruvoyltetrahydropterin synthase from *Drosophila melanogaster* [10] was a gift of J. J. Yim (Department of Microbiology, Seoul National University, Seoul, Korea) and had a specific activity of 3.3 nmol·min^{-1}·mg^{-1} as determined by a previously published method [11].

Sepiapterin reductase was partially purified from rat liver by a protocol modified from [12], all procedures were carried out at 4 °C; 50 g of rat liver was homogenized by means of an Ultra Turrax (IKA, Staufer, Germany) in 100 ml of 10 mM potassium phosphate buffer, pH 6.8, containing 100 mg/l of the protease inhibitor pefabloc [4-(2-aminoethyl)benzolsulphonyl fluoride hydrochloride, Merck]. After centrifugation (30 min, 13000 *g*), 10 ml of protamine sulphate [0.2% (w/v) in distilled water] was slowly added to the supernatant, the mixture was stirred for 5 min and the solid was removed by centrifugation. The supernatant was then applied in 10 ml portions to DEAE/f.p.l.c. Following equilibration for 10 min with 10 mM potassium phosphate, pH 6.8, a 30 min linear gradient to 10 mM potassium phosphate containing 300 mM KCl eluted the enzyme activity at 200 mM KCl. Active fractions were pooled and collected by precipitation with ammonium sulphate (38 g added to 100 ml of the solution). After stirring for 30 min, the precipitate was collected by centrifugation (15 min, 13000 *g*) and dissolved in 10 mM potassium phosphate, pH 6.8, containing 100 mg/l pefabloc. This was then subjected to hydrophobic interaction f.p.l.c. using the Fractogel TSK 650 Butyl column. At a flow rate of 1 ml/min, the column was washed for 10 min with 50 mM potassium phosphate buffer, pH 6.8, containing 200 g/l ammonium sulphate, followed by a 40 min linear gradient to 50 mM potassium phosphate. The enzyme activity eluted at 60 g/l ammonium sulphate. Active fractions were pooled, desalted to 10 mM potassium phosphate, pH 6.8, by means of PD-10 columns (Pharmacia), concentrated by ultrafiltration (cut-off 10 kDa, Centrisart, Sartorius, Göttingen, Germany) and subjected to gel-filtration f.p.l.c. on Superose 12, equilibrated at a flow rate of 0.5 ml/min with 50 mM potassium phosphate, pH 6.8, containing 150 mM KCl. The activity eluted corresponding to a molecular mass of 28–32 kDa. Sepiapterin reductase activity was determined in each fraction by monitoring the decrease of sepiapterin by u.v.-absorption at 405 nm in a microplate reader thermostated to 37 °C (Anthos 2001, Labtec, Salzburg, Austria) for 20 min. To 5 μl of the fraction, 10 μl of 1 mM sepiapterin was added and the reaction was started by addition of 100 μl of 0.2 mM NADPH (dissolved in 0.1 M potassium phosphate buffer, pH 6.8). The preparation obtained had a specific activity of 49 nmol·min^{-1}·mg^{-1}.

Enzymic synthesis of 6*R*-[3′-^3H]-5,6,7,8-tetrahydrobiopterin from [8,5′-^3H]GTP

A 500 μl volume of the [8,5′-^3H]GTP solution [500 μCi, 15 nmol, supplied in 50% (v/v) aqueous ethanol] was evaporated to dryness in a stream of nitrogen. A 100 μl volume of a mixture of GTP cyclohydrolase I (115 nmol/min), 6-pyruvoyltetrahydropterin synthase (0.5 nmol/min), sepiapterin reductase (6 nmol/min), dihydropteridine reductase (385 nmol/min), 4 mM NADPH, 10 mM dithioerythritol, 0.1 mM EDTA, 10 mM $MgCl_2$ and 0.1 M KCl in 0.1 M Tris/HCl, pH 7.5, was added and the mixture incubated for 60 min at 37 °C. The reaction mixture was then injected directly into the h.p.l.c. system for purification of the compound (see below).

Chemical synthesis of 6-*R*,S-[6-^3H]-5,6,7,8-tetrahydrobiopterin from 7,8-dihydrobiopterin by reaction with [^3H]$NaBH_4$

A 10 μl volume of 100 mM 7,8-dihydrobiopterin (1 μmol) was mixed with 82 μl of [^3H]$NaBH_4$ (38 nmol, 2 mCi, supplied in 0.01 M NaOH) for 2 min at room temperature. Potassium phosphate (8 μl, 1 M, pH 3.0) was added and the solution was stirred for an additional 2 min ($^3H_2$ is released at this step). The reaction mixture was then injected directly into the h.p.l.c. system to separate and isolate the 6*R*- and 6*S*- isomers of [6-^3H]-5,6,7,8-tetrahydrobiopterin. Neither prolongation of the reaction time (up to 120 min) nor alteration of the reaction temperature (up to 55 °C) increased the yield or the 6*R*/6*S* product ratio.

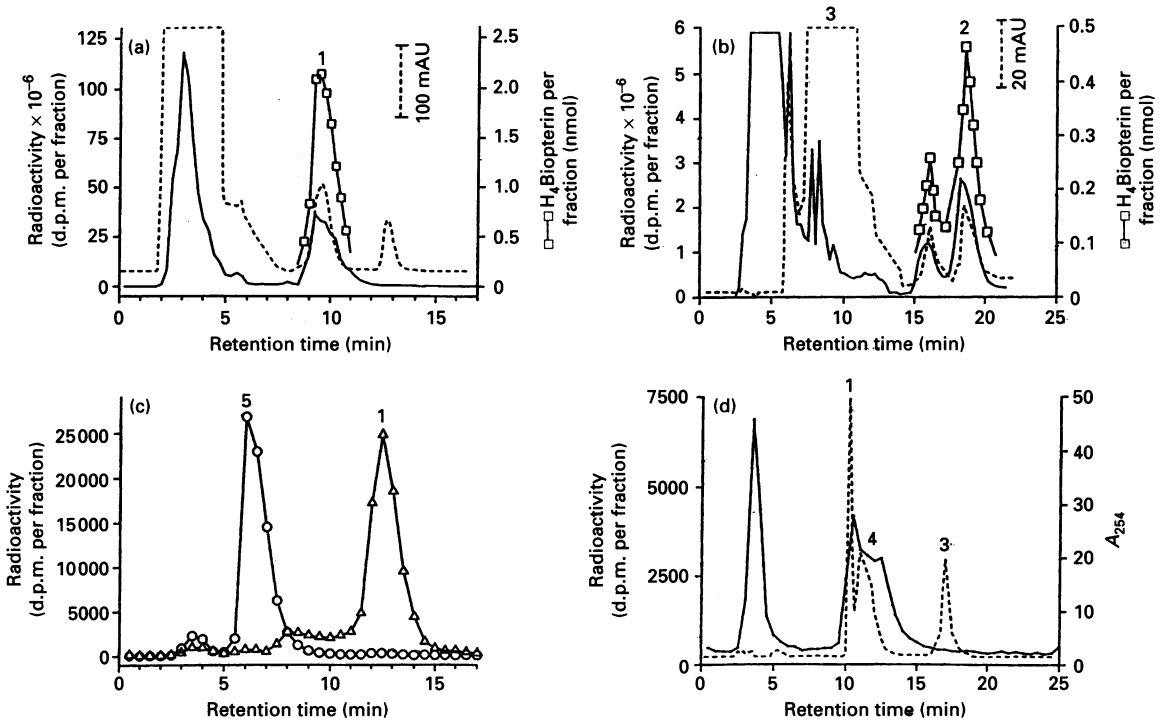

Figure 1 H.p.l.c. analysis of reaction mixtures of ^{3}H-labelled tetrahydrobiopterin

Reaction and separation conditions are detailed in the Materials and methods section. (**a**) Enzymic labelling mixture separated by ion-exchange h.p.l.c.; (**b**) chemical labelling mixture separated by ion-exchange h.p.l.c.; (**c**) separation of 6R-[3′-^{3}H]-5,6,7,8-tetrahydrobiopterin before (△) and after (○) oxidation with HCl/I_2 by ion-exchange h.p.l.c.; (**d**) products of aerobic oxidation of 6*R*-[6-^{3}H]-5,6,7,8-tetrahydrobiopterin separated by reverse-phase h.p.l.c. Continuous lines show radioactivity in d.p.m. per fraction, dotted lines u.v. absorption at 254 nm, □ show the tetrahydrobiopterin content of fractions measured as difference of biopterin subsequent to chemical oxidation in acid and base, respectively. Notation of peaks: 1, 6*R*-5,6,7,8-tetrahydrobiopterin; 2, 6*S*-5,6,7,8-tetrahydrobiopterin; 3, 7,8-dihydro-biopterin; 4, 6,7(8*H*)dihydrobiopterin; 5, biopterin.

H.p.l.c. of the reaction mixtures

H.p.l.c. of the reaction mixtures was performed by eluting the Nucleosil 10 SA cation-exchange column with 100 mM potassium phosphate, pH 3.0, containing 5 mM dithioerythritol at a flow rate of 0.8 ml/min and an injection volume of 100 μl. Fractions were collected every 15 s. For the preparative runs using labelled material, the u.v.-detector lamp was switched off to minimize decomposition of the desired products. The absorption profiles shown in the Figures originate from parallel experiments using unlabelled material under identical conditions. Radioactivity was determined in 2 μl aliquots of the fractions by use of a scintillation counter (LS3801, Beckman, Palo Alto, CA, U.S.A.). The tetrahydrobiopterin content in fractions was determined by oxidation with iodine in HCl to obtain the sum of all biopterin species or in NaOH for the determination of all species except tetrahydrobiopterin [13], using a previously described protocol [14]. Briefly, 10 μl aliquots of the fractions were diluted with 90 μl of distilled water. A 5 μl volume of 1 M HCl or 5 μl of 1 M NaOH and 5 μl of 0.1 M I_2 in 0.25 M KI were added. After incubation for 1 h in the dark, 10 μl of HCl was added to the alkaline solution only and 10 μl of ascorbic acid was added to both incubations to destroy excess iodine. Biopterin was then quantified by reverse-phase h.p.l.c. as follows. The 250 mm RP-18 column was equilibrated with 15 mM potassium phosphate, pH 6.4, at a flow rate of 0.8 ml/min. Biopterin was detected by fluorescence (excitation λ 350 nm, emission λ 440 nm). For the analysis of 6,7(8*H*)-dihydrobiopterin, the 250 mm RP-18 column was eluted with 50 mM potassium phosphate, pH 7.7, and no dithioerythritol was included in the elution buffer. In the case of ion-exchange chromatography of labelled derivatives, a 20 min elution with 0.5 M potassium phosphate, pH 6.8, was included between each sample to clear the column of all radioactive material.

RESULTS

We employed two different approaches to synthesize ^{3}H-labelled tetrahydrobiopterin (Scheme 1). The enzymic approach (Scheme 1A) starts from [8,5′-^{3}H]GTP [I], which is incubated with GTP cyclohydrolase I (*a*), 6-pyruvoyltetrahydropterin synthase (*b*) and sepiapterin reductase (*c*) in the presence of Mg^{2+} and NADPH. Whereas the ^{3}H-label at position 8 of GTP is lost in the GTP cyclohydrolase I reaction (*a*), the ^{3}H-label at 5′ is incorporated into 6*R*-tetrahydrobiopterin at position 3′ [IV]. This is the only carbon-bound hydrogen of GTP which is incorporated into tetrahydrobiopterin, since downstream of 7,8-dihydroneopterin triphosphate [II] the 3′ and 4′ hydrogen atoms of the ribose moiety of GTP are lost in the formation of 1′ and 2′ keto groups of 6-pyruvoyltetrahydropterin [III]. The chemical approach (Scheme 1B) employs a reduction of the 5,6 double bond of 7,8-dihydrobiopterin [V] by [^{3}H]$NaBH_4$, which yields 6-*R*,*S*-[6-^{3}H]-5,6,7,8-tetrahydrobiopterin [VI]. This label is stable to initial oxidation to the 'quinonoid' 6,7(8*H*)-dihydrobiopterin [VII], but is lost upon the rearrangement of this labile compound to 7,8-dihydrobiopterin [V] (see also below).

The u.v.-absorption h.p.l.c. profiles derived from incubations with unlabelled material showed that the tetrahydrobiopterin

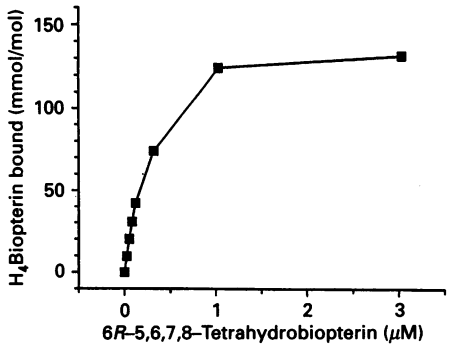

Figure 2 Binding of 6*R*-[3′-^{3}H]-5,6,7,8-tetrahydrobiopterin to brain nitric-oxide synthase

Brain nitric-oxide synthase (2–4 μg, purified as detailed in [15]) was incubated for 10 min at 37 °C with a fixed concentration of 6*R*-[3′-^{3}H]-5,6,7,8-tetrahydrobiopterin (20 nm, 18 nCi) and increasing concentrations of unlabelled 6*R*-5,6,7,8-tetrahydrobiopterin in 0.1 ml of triethanolamine/HCl buffer, pH 7.0, containing 0.1 mM NADPH and 10 μM dithiothreitol. Samples were assayed for binding of labelled material by polyethylene glycol precipitation, rapid vaccum filtration over Whatman glass fibres (GF/B), and determination of radioactivity retained on the filters. Non-specific binding, determined in the presence of 1 mM unlabelled 6*R*-5,6,7,8-tetrahydrobiopterin was < 3% at equilibrium (incubation time > 6 min) and subtracted from total binding. Data are representative of six similar experiments.

products were well separated from the starting materials (Figure 1a and 1b). In the labelled incubations, radioactivity incorporated into tetrahydrobiopterin was also well separated from other radioactive components of the reaction mixture (Figures 1a and 1b). With the enzymic approach 25% of the total radioactivity was incorporated into the product, which, as expected, was the 6*R*-isomer only (Figure 1a). A specific activity of 9.24 ± 1.89 Ci/mmol (mean ± S.D., $n = 11$) was achieved. Assuming that the activity in the starting material GTP was evenly distributed among the 8- and 5′-positions, this corresponds to 50% of the theoretical yield. The resulting solution of [3′-^{3}H]-5,6,7,8-tetrahydrobiopterin had a radiochemical purity of more than 95% on ion-exchange- and on reverse-phase-h.p.l.c. More than 95% of the biopterin was present as tetrahydro derivative.

In the chemical labelling experiments, only 1.2% of the total radioactivity was incorporated into the two products 6*R*- and 6*S*-[6-^{3}H]-5,6,7,8-tetrahydrobiopterin (Figure 1b). The 6*R*-isomer made up 30% and the 6*S*-isomer 70% of the tetrahydrobiopterin products. The specific activities obtained were 2.35 ± 0.16 Ci/mmol for 6*R*- and 2.48 ± 0.34 Ci/mmol for 6*S*-[6-^{3}H]-5,6,7,8-tetrahydrobiopterin (mean ± S.D., $n = 4$). The tetrahydrobiopterin content of the collected fractions of 6*R*- and 6*S*-[6-^{3}H]-5,6,7,8-tetrahydrobiopterin was 66 ± 4% and 77 ± 6% of total biopterin, respectively ($n = 3$, mean ± S.D.).

When [3′-^{3}H]-5,6,7,8-tetrahydrobiopterin was subjected to oxidation with iodine in HCl, the label was kept in the product to yield [3′-^{3}H]biopterin (Figure 1c). Aerobic oxidation of 6R-[6-^{3}H]-5,6,7,8-tetrahydrobiopterin at pH 7.7 in the absence of the antioxidant dithioerythritol first yielded [6-^{3}H]-6,7(8*H*)-dihydrobiopterin. Upon rearrangement of this labile compound to 7,8-dihydrobiopterin, the label was lost (Figure 1d). Detailed quantitative analysis showed that upon oxidation of 6*R*-[6-^{3}H]-5,6,7,8-tetrahydrobiopterin with stoichiometric amounts of dichlorophenol-indophenol [which initially yields 6,7(8*H*)-dihydrobiopterin] and immediate reduction back to 5,6,7,8-tetrahydrobiopterin by an excess of dithioerythrol, no label is lost from the product (recovery 97.2% ± 11.5%, $n = 3$, mean ± S.D.).

The enzymically synthesized [3′-^{3}H]-5,6,7,8-tetrahydrobiopterin was used for binding studies with purified brain nitric-oxide synthase. Figure 2 shows that binding of [3′-^{3}H]-5,6,7,8-tetrahydrobiopterin was concentration-dependent. Brain nitric-oxide synthase already contains about 0.25 mol tetrahydrobiopterin per mol subunit. At the enzyme concentration employed (3 μg/ml), however, this would contribute less than 5 nM tetrahydrobiopterin so that no correction for this contribution was necessary. A 140 mmol amount of the radioligand bound per mol of nitric-oxide synthase when a subunit molecular mass of 160 kDa [15] is assumed. However, as observed previously for binding of ^{3}H-labelled N^G-nitro-L-arginine to brain nitric-oxide synthase [16], the amount of bound radioligand may be underestimated in the binding assay due to low counting-efficiency of radioactivity retained on the filters. Details of the binding-kinetics and the influence of substrate and inhibitor on the binding of tetrahydrobiopterin to nitric-oxide synthase will be presented elsewhere [17].

DISCUSSION

Using the three biosynthetic enzymes GTP cyclohydrolase I, 6-pyruvoyltetrahydropterin synthase and sepiapterin reductase, we were able to incorporate the ^{3}H-label of position 5′ of GTP into position 3′ of 6*R*-tetrahydrobiopterin. This only carbon-bound hydrogen of GTP incorporated into tetrahydrobiopterin in this biosynthesis is placed adjacent to a carbonyl function in the 6-pyruvoyltetrahydropterin intermediate ([III] in Scheme 1A). Fortunately, sepiapterin reductase reduces this carbonyl before the 3′ label is lost by keto–enol tautomerization. Even though only one hydrogen can be used to label tetrahydrobiopterin in this way, a product with much higher specific activity (9.2 Ci/mmol, the present work) than with the use of [U^{14}-C]GTP (0.46 Ci/mmol, [18]) is obtained.

The enzymically formed 6*R*-[3′-^{3}H]-5,6,7,8-tetrahydrobiopterin is also superior in its specific activity, radiochemical yield and purity to the 6-^{3}H-labelled products derived from chemical synthesis, which we found to have a specific activity of 2.4 Ci/mmol. The reduction of biopterin with $NaBH_4$ is known to proceed only at high excess of $NaBH_4$ [19]. We optimized the reaction conditions to get a maximum of tetrahydrobiopterin at a given amount of $NaBH_4$ at the concentration and in the solvent in which the radiolabelled derivative is available. Although the reaction with [^{3}H]$NaBH_4$ proceeds only to an incorporation of 1.2% of the radioactivity into tetrahydrobiopterin, we were able to isolate solutions of small amounts of 6*S*- and 6*R*-[6-^{3}H]-5,6,7,8-tetrahydrobiopterin in this way. Analysis of behaviour to oxidation clearly shows that the label is only present in the 6-position, since formation of the 'quinonoid' 6,7(8*H*)-dihydrobiopterin ([VII] in Scheme 1B) and immediate reduction does not lead to a loss of the label. Thus, in contrast to a previous assumption [7], the ^{3}H at the nitrogen in position 5 initially formed in the hydrogenation reaction is rapidly exchanged with water. In addition to the higher yield of the synthesis, the enzymically formed 6*R*-[3′-^{3}H]-5,6,7,8-tetrahydrobiopterin offers the additional advantage over 6*R*-[6-^{3}H]-5,6,7,8-tetrahydrobiopterin that the side-chain label is retained upon oxidation to 7,8-dihydrobiopterin and biopterin. This is of particular importance, e.g. for the study of uptake and distribution of tetrahydrobiopterin in cells or animals. 7,8-Dihydrobiopterin, which is formed rapidly from tetrahydrobiopterin supplied to culture media, may be taken up by cells and reduced back to tetrahydrobiopterin by dihydrofolate reductase [20]. Only the 3′-^{3}H- but not the 6-^{3}H-label is then retained in tetrahydrobiopterin.

6*R*-[3′-^{3}H]-5,6,7,8-Tetrahydrobiopterin proved useful for

studying the binding of tetrahydrobiopterin to nitric-oxide synthase. In addition, it will be a valuable tool to investigate e.g. transport of tetrahydrobiopterin across membranes and to study the kinetics and metabolism of tetrahydrobiopterin applied *in vivo*, its localization in cell compartments and in tissue sections.

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