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Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine

(arginine monooxygenase/cyclic GMP/methotrexate/dihydrofolate reductase/dihydropteridine reductase)

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ABSTRACT **Studies with purified nitric oxide synthase from rat cerebellum have confirmed previous reports that product formation is enhanced by tetrahydrobiopterin [H_4B; 6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin]. The effect of the natural isomer, (6*R*)-H_4B, is observed at extremely low (<0.1 μM) concentrations and is remarkably selective. At these concentrations, only the diastereoisomer (6*S*)-H_4B, the structural isomer 7-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin, and 7,8-dihydrobiopterin showed detectable effects. Our observations are inconsistent with a stoichiometric role for H_4B in the oxygenation of arginine [e.g., Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L. & Wiseman, J. (1991) *J. Biol. Chem.* 266, 6259–6263]. Activity is initially independent of added H_4B; enhanced product formation with H_4B is observed only as incubation progresses. The effect of H_4B is catalytic, with each mole of added H_4B supporting the formation of >15 mol of product. Recycling of H_4B was excluded by direct measurement during nitric oxide synthesis and by the demonstration that nitric oxide synthase is not inhibited by methotrexate. These combined results exclude H_4B as a stoichiometric reactant and suggest that H_4B enhances product formation by protecting enzyme activity against progressive loss. Preliminary studies indicate that the decreased activity in the absence of added H_4B does not depend on catalytic turnover of the enzyme. The role of H_4B may be allosteric or it may function to maintain some group(s) on the enzyme in a reduced state required for activity.**

Nitric oxide synthase (NOS) catalyzes the oxygenation of arginine in the presence of NADPH to form nitric oxide, citrulline, and $NADP^+$. The enzyme is of great interest because nitric oxide appears to participate in a variety of physiological processes. In addition to its classic role as the endothelium-derived relaxing factor in mediating vasodilation (1–3), nitric oxide has been implicated in regulating macrophage antitumor and antimicrobial activity, platelet adhesion, and cerebellar signaling (4). Nitric oxide stimulates guanylate cyclase, yielding increased production of cyclic GMP that is proposed to mediate cerebellar signaling and possibly other physiological effects of nitric oxide (4). NOS has been reported in a variety of mammalian tissues (4). Differences in cofactor, substrate, and inhibitor specificities suggest that NOS may exist in at least three distinct forms (4–6).

Tetrahydrobiopterin [H_4B; 6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin] causes a marked and specific stimulation of macrophage NOS (7, 8). The original studies of Bredt and Snyder (9) of purified brain (cerebellar) NOS did not include the effects of H_4B. However, recent studies show that activity of cerebellar NOS is also increased by H_4B (10, 11). The biochemical basis for this effect is poorly understood. Studies of the macrophage enzyme (7, 12, 13) have been interpreted as showing that H_4B participates stoichiometrically in the reaction; i.e., it provides reducing equivalents required for the overall oxygenation of arginine to nitric oxide and citrulline.

The present studies on purified NOS from rat cerebellum show that this is not the case for the brain enzyme. H_4B is shown to preserve NOS activity *in vitro* by preventing the progressive loss of activity that otherwise proceeds in the absence of this factor. The effect is observed at extremely low (<0.1 μM) concentrations of H_4B and is remarkably selective for this cofactor.

MATERIALS AND METHODS

Materials. L-[U-^{14}C]Arginine was obtained from NEN or Amersham. L-[2,6-^{3}H]Phenylalanine and L-[U-^{14}C]tyrosine were obtained from Amersham. (6*R*)-H_4B, (6*S*)-H_4B, tetrahydroneopterin [6-(L-*erythro*-1,2,3-trihydroxypropyl)-5,6,7,8-tetrahydropterin], 7-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin [(7*R*,*S*)-H_4B], 6-methyl-5,6,7,8-tetrahydropterin, 6,7-dimethyl-5,6,7,8-tetrahydropterin, 7,8-dihydrobiopterin (H_2B), 7,8-dihydroneopterin, and L-biopterin were obtained from B. Schircks Laboratories (Jona, Switzerland). Glucose-6-phosphate dehydrogenase (type 23) from *Leuconostoc mesenteroides* was obtained from Sigma; 1 unit of activity oxidizes 1 μmol of glucose 6-phosphate in the presence of $NADP^+$ at pH 7.8 and 30°C. Dihydropteridine reductase (DHPR), EC 1.6.99.7, from sheep liver was purified to the stage of elution from calcium phosphate gel (14). Phenylalanine hydroxylase (PAH), EC 1.14.16.1, was prepared by what has been described as the "hybrid" procedure (15). Bovine liver dihydrofolate reductase (DHFR), EC 1.5.1.3, was obtained from Sigma and was desalted immediately before use on a column of Sephadex G-25 equilibrated with 50 mM potassium phosphate (pH 6.5). All other materials were obtained from Sigma.

Assay of NOS. Activity was measured essentially as described (9) by monitoring the conversion of [^{14}C]arginine to [^{14}C]citrulline. The reaction mixture (150 μl) at 25°C contained enzyme, L-[^{14}C]arginine (86.9 nCi; 1 nCi = 37 Bq) at the concentration specified, 250 mM Hepes (pH 6.4 with KOH), 10 μM NADPH, 0.60 mM EDTA, 0.83 mM $CaCl_2$, 150 μg of bovine serum albumin (BSA), 1300 units (20 μg) of

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Abbreviations: BSA, bovine serum albumin; NOS, nitric oxide synthase; DHPR, dihydropteridine reductase; DHFR, dihydrofolate reductase; PAH, phenylalanine hydroxylase; MTX, methotrexate; GSH, reduced glutathione; 2′,5′-ADP, adenosine 2′,5′-bisphosphate; H_4B, 6-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin (tetrahydrobiopterin); H_2B, 6-(L-*erythro*-1,2-dihydroxypropyl)-7,8-dihydropterin; (7)-H_4B, 7-(L-*erythro*-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropterin.

catalase, 2 μg of calmodulin, and H_4B as specified. Except for the study on pteridine specificity (Table 1), (6*R*)-H_4B was used. [^{14}C]Citrulline in the reaction product was determined as described (9) by chromatography on AG 50W-X8, 200–400 mesh, Na^+ form (Bio-Rad).

H_4B Recycling Assay. A sensitive assay for H_4B recycling was developed based on coupling of H_4B synthesis to the H_4B-dependent hydroxylation of phenylalanine to tyrosine catalyzed by PAH. All assay mixtures (50 μl) contained the following reactants (basic reaction mixture): L-[^{3}H]phenylalanine (200 μM, 34.8 μCi), purified PAH from rat liver with activity equivalent to 39 nmol/min determined with the colorimetric assay (15), 25 mM glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 433 units (6.7 μg) of catalase, 0.05 μM (6*R*)-H_4B, and 0.1 mM lysolecithin to increase PAH activity in the presence of H_4B (16). To maximize detection of H_4B recycling, assays were conducted under three conditions (A, B, and C). For condition A, which approximated as closely as possible the conditions used for assay of NOS, the following components were added to the basic reaction mixture described above: 250 mM Hepes (pH 6.4 with KOH), 0.6 mM EDTA, 0.82 mM $CaCl_2$, 0.73 μg of calmodulin, 10 μM NADPH, 6.9 μM L-arginine, and 50 μg of BSA. For condition B, which approximated conditions optimal for recycling of H_4B in the colorimetric assay of PAH (15), 100 mM potassium phosphate (pH 6.8) and 10 μM NADH were added to the basic reaction mixture. Condition C was the same as B except that NADPH was substituted for NADH. Enzyme was added as shown. Incubations were at 25°C for 30 min and were stopped by addition of 4.5 μl of 60% (wt/vol) trichloroacetic acid. Samples were centrifuged and aliquots of the supernatant solutions were mixed with nonradioactive carrier phenylalanine (25 nmol) and tyrosine (10 nmol) plus an amount of [^{14}C]tyrosine to yield a $^3H/^{14}C$ ratio of ≈10 for the tyrosine crystals described below. The product of the reaction, [^{3}H]tyrosine, was purified by HPLC of this mixture on a reverse-phase column [Hypersil ODS (Hewlett–Packard), 60 × 4.6 mm], using isocratic elution with 30 mM sodium acetate, pH 7/3% methanol (17). Fractions enriched in [^{3}H]tyrosine (detected with [^{14}C]tyrosine marker) were mixed with 100 mg of L-tyrosine and further purified by recrystallization (twice) to a constant ratio of ^3H to ^{14}C. This ratio was used to calculate the amount of [^{3}H]tyrosine product. All assays were corrected for activity measured in a corresponding reaction mixture containing 5% trichloroacetic acid.

Purification of NOS. Except for the modifications noted, NOS was purified by the method of Bredt and Snyder (9). Frozen male Sprague–Dawley rat cerebella from Bioproducts for Science (Indianapolis) were used. The crude supernatant from frozen cerebella gave the same level of NOS activity as fresh cerebella. The number of rat cerebella was increased 4-fold to 72, and the volume of the DEAE-Sephacel (Pharmacia) column was increased from 20 ml to 30 ml (2.5-cm internal diameter). The active fractions obtained from DEAE-Sephacel chromatography were applied to a column of adenosine 2′,5′-bisphosphate (2′,5′-ADP)-Sepharose 4B and eluted with a buffer containing 10 mM NADPH (9). This eluate was concentrated first in a Centricell of 30,000 nominal molecular weight limit (Polysciences), and then in a Centricon-30 (Amicon) concentrator to a final volume of ≈100 μl. Prior to use, both concentrators were preblocked with NOS from earlier purifications, to minimize loss of protein by adsorption, and then washed with 10 mM Tris·HCl, pH 7.4/1 mM EDTA/20% (vol/vol) glycerol. The concentrated enzyme was then diluted with 2 ml of this buffer and again concentrated to ≈100 μl. This step was repeated twice to yield an ≈80-fold concentrated preparation that was essentially free of the NADPH and mercaptoethanol originally present in the eluate. All procedures were carried out at 4°C, unless stated otherwise, and completed within 15 hr of the initial homogenization of cerebella. Protein was determined by the method of Bradford (18) with the use of Coomassie protein assay reagent (Pierce) and BSA as standard. Specific activities determined on preparations obtained immediately after elution from 2′,5′-ADP-Sepharose 4B ranged from 80 to 800 nmol/min per mg of protein. A comparable range of specific activities of purified rat cerebellar NOS has also been observed by other workers: 107 nmol/min per mg by Schmidt *et al.* (11) and 960 nmol/min per mg by Bredt and Snyder (9). Decreases in specific activity of as much as 75% were sometimes observed during the concentration–washing step. One possible explanation for this variation and loss in specific activity is the extreme instability at 4°C of dilute solutions of the purified enzyme observed by us and by other workers (9, 11, 19). The concentrated enzyme was flash frozen in liquid nitrogen in 5-μl samples and stored at −70°C. Immediately (within 10–12 min) before assay, samples were thawed and diluted in 10 mM Tris·HCl, pH 7.4/1 mM EDTA/20% glycerol/0.1% BSA. Activity of frozen samples assayed in this way was approximately half that of freshly prepared samples, and this activity was retained for at least 6 weeks at −70°C.

RESULTS

To distinguish between effects on initial rate and stability of enzyme, cofactor requirements were determined at incubation times of 5 and 20 min. At each incubation time, NOS activity showed an absolute dependence on calmodulin, Ca^{2+}, and NADPH. NADH could not replace NADPH. Omission of H_4B reduced the rate to 57% and 43% of that with the complete reaction at 5 min and 20 min, respectively. No significant effect was observed by omission of BSA, EDTA, or catalase. Addition of FAD (2 μM), FMN (2 μM), or dithiothreitol (2.5 mM) did not affect the rate. Addition of reduced glutathione (GSH, 5 mM) decreased the rate to 59% and 42% of that with the complete reaction at 5 and 20 min, respectively. The effect of GSH plus FAD was similar to that of GSH alone.

The effect of H_4B in stimulating NOS could not be duplicated by a variety of other reductants. Thus, compared with a 300% increase in product formation in 30 min observed with 0.2 μM H_4B, no increase was observed with equimolar ascorbate or NADH. Replacement of H_4B with equimolar thiol (dithiothreitol, 2-mercaptoethanol, or GSH) actually inhibited product formation to ≈65% of that in the absence of added reductant.

Of a range of pterins studied (Table 1), highest activity was observed with (6*R*)-H_4B, the natural isomer of H_4B. Smaller increases were obtained with (6*S*)-H_4B, (7*R*,*S*)-H_4B, and H_2B. Biopterin was without effect. The following pterin derivatives at 0.2 μM were also without significant effect: 6,7-dimethyl-5,6,7,8-tetrahydropterin, 6-methyl-5,6,7,8-tetrahydropterin, tetrahydrofolate, tetrahydroneopterin, and dihydroneopterin. At relatively high concentrations (100 μM)

Table 1. Selectivity of H_4B in increasing NOS activity

Pterin	Relative activity, %*			
	0.05 μM	0.2 μM	1 μM	10 μM
(6*R*)-H_4B	179 ± 7	199 ± 7	225 ± 9	244 ± 2
(6*S*)-H_4B	106 ± 2	120 ± 3	136 ± 1	160 ± 1
(7*R*,*S*)-H_4B	—	131 ± 5	—	—
7,8-H_2B	117 ± 3	128 ± 4	121 ± 2	123 ± 3
Biopterin	105 ± 3	100 ± 3	94 ± 4	87 ± 1

All reaction mixtures contained the components described for the standard NOS assay with 6.9 μM [^{14}C]arginine and 0.33 μg of NOS.
*Mean ± SD of duplicate determinations are expressed as a percentage of the control value (0.56 μM citrulline, 30 min) with no added pterin.

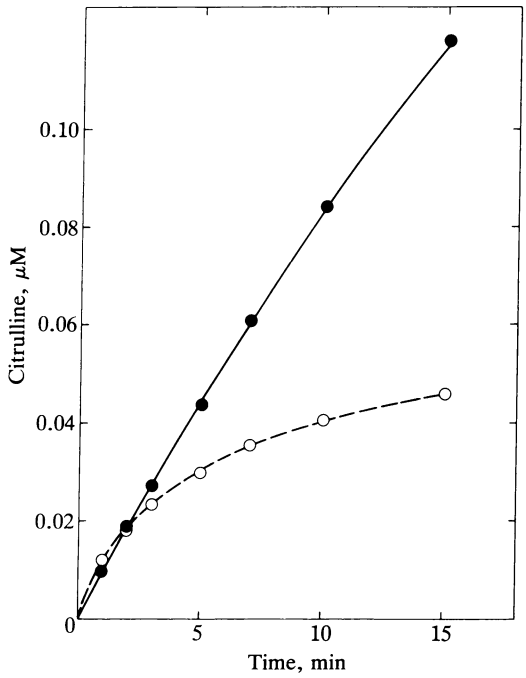

FIG. 1. Time course of NOS in the presence or absence of H_4B. Reaction mixtures contained 1.9 μM [^{14}C]arginine and the components described in the standard assay of NOS, with addition of 60 μM H_4B (●) or with no added H_4B (○). The concentration of NOS (1 nM) was approximated from the protein content of the purified preparation (0.04 μg) by assuming a native molecular mass of 239.5 kDa. The latter value is the mean of the reported values of 200 kDa (9) and 279 kDa (11) for rat cerebellar NOS.

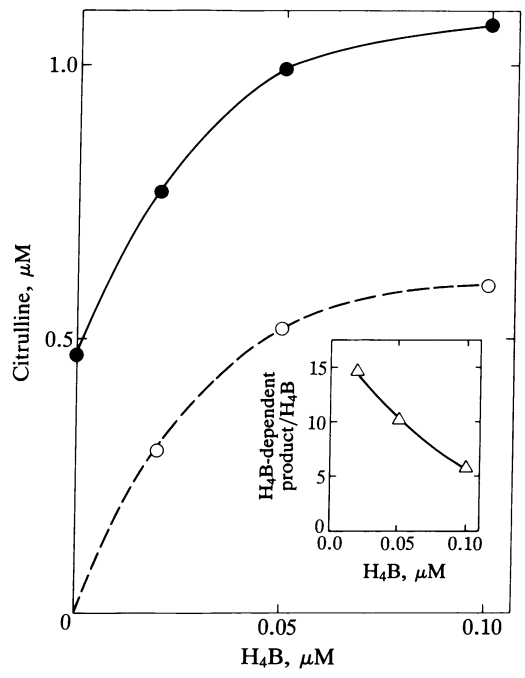

FIG. 2. Effect of H_4B concentration on NOS activity. Reaction mixtures contained 0.06 μg of NOS, 3.45 μM [^{14}C]arginine, and H_4B as shown. Incubations were for 15 min. ●, Total product formation; ○, H_4B-dependent product formation. (*Inset*) Data from the lower curve are plotted to show moles of H_4B-dependent product per mole of added H_4B as a function of H_4B concentration.

of 6-methyl-5,6,7,8-tetrahydropterin, stimulation was ≈40% of the maximum stimulation observed with (6*R*)-H_4B.

Fig. 1 illustrates a more detailed study of the time course of activity in the presence or absence of H_4B. The reaction is independent of added H_4B over the first few minutes, during which time the enzyme turns over at maximal rate ≈18 times (0.018 μM citrulline/0.001 μM enzyme) in the absence of added H_4B. Rates in the absence of H_4B progressively decline after 2 min, while that in the presence of H_4B remains relatively constant. The net effect is that the apparent stimulation in rate by H_4B becomes progressively greater with incubation time.

The ability of the reaction to proceed optimally in the absence of H_4B during early incubation times suggests that H_4B is not reacting stoichiometrically in nitric oxide synthesis but rather acting to prevent progressive loss of enzyme activity with increasing incubation time. Further evidence for this proposal is provided in Fig. 2, which illustrates the effects of H_4B concentration on total product formation (upper curve) and H_4B-dependent product formation (lower curve). H_4B is effective at very low concentrations, with half-maximum stimulation being obtained at only 0.02 μM H_4B. Each mole of added H_4B can support the formation of many moles of product (Fig. 2 *Inset*). For example, extrapolation of the curve to lower concentrations of H_4B indicates that 1 mol of H_4B can catalyze the formation of >15 mol of product.

These combined results do not exclude the possibility that H_4B is a stoichiometric reactant in nitric oxide synthesis if the proposed product of such a reaction, H_2B, is recycled to H_4B. Studies with the aromatic amino acid hydroxylases show that the dihydropterin product formed initially is the quinonoid form of H_2B (20). This product can be recycled to H_4B either chemically, in the presence of high concentrations of reductants such as ascorbate, thiols, reduced pyridine nucleotides, or enzymically, in the presence of DHPR (21). Methylenetetrahydrofolate reductase (E.C. 1.1.99.15) also catalyzes reduction of quinonoid H_2B to H_4B, but at a lower rate than that by DHPR (22). Quinonoid H_2B subsequently rearranges to 7,8-H_2B (and 7,8-dihydropterin) (23, 24). DHFR is required for recycling of 7,8-H_2B to H_4B (25). In our experiments, any chemical regeneration of H_4B was minimized by omission of thiol compounds and by maintaining a low concentration (10 μM) of NADPH in the assay. The studies described below were conducted to provide direct evidence against recycling of H_4B either chemically or enzymically.

Table 2 compares NOS activities and H_4B recycling activities of a purified preparation of NOS. Recycling of H_4B was measured by coupling any formation of this compound to the conversion of phenylalanine to tyrosine catalyzed by PAH. To maximize detection, H_4B was assayed under three conditions with either NADPH or NADH (see *Materials and Methods*). NADPH is the preferred substrate for DHFR (26) and methylenetetrahydrofolate reductase (22), whereas NADH is preferred for DHPR (14). Bovine liver has been

Table 2. Comparison of NOS activity and H_4B recycling of purified NOS

	NOS activity, pmol of citrulline			H_4B recycling, pmol of tyrosine		
Exp.	− H_4B	+ H_4B	Δ*	A	B	C
1	176	322	146	—	≤2.5	—
2	175	232	57	≤2.5	—	≤2.5

Mixtures for assays of NOS and H_4B recycling contained 0.12 μg of purified NOS incubated for 30 min. H_4B (0.05 μM; 2.5 pmol in a final volume of 50 μl) was added to all H_4B recycling assays. Where specified, H_4B (0.05 μM; 7.5 pmol in a final volume of 150 μl) was added for NOS assays. The concentration of [^{14}C]arginine in the NOS assay was 6.9 μM. Conditions for assay of H_4B recycling were as follows (see *Materials and Methods* for details): A, conditions similar to NOS assay; B, conditions optimal for recycling of H_4B; C, same as B except NADPH was substituted for NADH.
*Increase in citrulline formation due to addition of 7.5 pmol of H_4B.

Table 3. Absence of inhibition of NOS by MTX

Pterin added	Relative activity, %	
	Control	+ MTX
None	100	104
H_4B	305	302
H_2B	150	158
H_4B plus H_2B	221	205

Values are means of two determinations, expressed as a percentage of the control value (1.1 μM citrulline, 20 min) determined with no added pterin. Standard deviations were ≤8%. Assays contained 0.41 μg of NOS and 6.9 μM [^{14}C]arginine. Pterins were added at 10 μM and MTX at 100 μM.

reported to contain two distinct species of DHPR, one of which resembles the majority of mammalian DHPR enzymes in being relatively specific for NADH, while the other is specific for NADPH (27). Positive control experiments established that product formation was linear with DHPR to at least 75 pmol of tyrosine per 30 min, and for DHFR to at least 200 pmol of tyrosine per 30 min. In the absence of enzyme, the amount of tyrosine formed (≤2.5 pmol) was at the limit of detection of the assay.

The relative ability of NOS to catalyze citrulline synthesis and H_4B recycling (tyrosine synthesis) was compared in two experiments (Table 2). In the first, addition of H_4B (7.5 pmol) resulted in an increased synthesis of 146 pmol of citrulline, most of which (146 − 7.5 = 138.5 pmol) would have to be derived from recycling if H_4B were a stoichiometric component of the reaction. No H_4B recycling was detected above that expected to be formed from the 2.5 pmol of added H_4B. Any H_4B recycled would therefore have been <2% {[2.5/(146 − 7.5)] × 100} of that required for stoichiometric reaction of H_4B. In the second experiment, a comparable value of 5% {[2.5/(57 − 7.5)] × 100} was obtained when H_4B recycling was assayed under conditions A and C instead of condition B.

Further evidence against recycling of H_4B was provided by studies of the effects of methotrexate (MTX) on NOS activity. MTX is an extraordinarily potent inhibitor of DHFR, with a K_i in the nanomolar range (28). MTX at much higher concentrations causes comparable inhibition of DHPR (14) and methylenetetrahydrofolate reductase (29, 30). Separate experiments (data not shown) confirmed that 100 μM MTX caused ≈70% inhibition of DHPR when the enzyme was assayed by the recycling assay with either NADH or NADPH, in good agreement with the reported K_i value of 38 μM (14). Table 3 shows the effects of 100 μM MTX on NOS activity measured in the absence or presence of pterin. No significant inhibition of citrulline synthesis by MTX was observed under any of the conditions tested. An additional finding was that activity with H_4B and H_2B was intermediate between that with either pterin alone.

Preliminary studies suggest that loss of activity in the absence of H_4B is not dependent on catalytic turnover of the enzyme. In these studies, NOS was preincubated in the presence or absence of H_4B under conditions either allowing or precluding catalytic turnover. After preincubation, any omitted component was restored to the reaction mixture and NOS activity was determined. The results of Table 4 show comparable NOS activity by omission of H_4B from the preincubation even without enzyme turnover.

Table 4. Effect of enzyme turnover on NOS activity during preincubation in the absence of H_4B

Exp.	NOS activity, μM			
	Preincubated with H_4B		Preincubated without H_4B	
	Turnover	No turnover	Turnover	No turnover
1	0.315	0.338	0.224	0.221
2	0.138	0.113	0.052	0.072

NOS was preincubated for 8 min (Exp. 1) or 20 min (Exp. 2) under the conditions of the standard assay with 6.9 μM [^{14}C]arginine, with the following modifications. H_4B (60 μM) was present as specified. Catalytic turnover was either prevented by omission of NADPH, calmodulin, Ca^{2+}, and EDTA or allowed to proceed by inclusion of these factors. At the end of the preincubation, a sample was removed for determination of [^{14}C]citrulline. Any omitted component was restored to the remaining reaction mixture and incubation was continued for an additional 12 min (Exp. 1) or 10 min (Exp. 2). The difference between the amount of [^{14}C]citrulline formed at the end of this period and the amount formed at the end of preincubation is a measure of the activity of NOS at the end of the preincubation. Reaction mixtures of Exp. 1 contained the equivalent of 0.38 μg of NOS (in 50 μl), and those of Exp. 2 contained 0.45 μg of NOS. Values for Exp. 1 are single determinations. The range determined from the duplicate sets of values in Exp. 2 did not exceed 12% of the reported mean values.

DISCUSSION

The absolute requirements observed for NADPH, Ca^{2+}, and calmodulin are in agreement with previous studies on the cerebellar enzyme (9, 10). Further, NADH will not substitute for NADPH. FAD and GSH were found to be necessary for maximum activity in a NOS system from macrophages (8). By contrast, no increase in activity was observed in our system by addition of FAD, FMN, dithiothreitol, GSH, or a combination of FAD and GSH. Hope *et al.* (31) have recently reported that NOS from rat brain is identical with the flavoprotein neuronal NADPH diaphorase. Based on this report, the absence of stimulation of cerebellar NOS by added flavin suggests that our isolated enzyme contains tightly bound flavin. While many of our preparations showed responses to thiols similar to those reported, some preparations exhibited a marked dependence on thiol, with maximum activity requiring both thiol and H_4B. A possible basis for the variable effects of thiol observed with different enzyme preparations will be discussed in a later publication.

The enhancement of product formation by H_4B addition showed a very high affinity (Fig. 2) and selectivity (Table 1) for this factor. Most remarkable is the very high structural and stereo selectivity for the pterin. Even the closely related structural isomer (7)-H_4B, the diastereoisomer (6*S*)-H_4B, and 6-methyl-5,6,7,8-tetrahydropterin were significantly less active than (6*R*)-H_4B. A marked selectivity for H_4B has also been reported for macrophage NOS (12). The small but significant stimulation by H_2B (Table 1) and its combined effect with H_4B (Table 3) has a special significance, which is discussed below.

A major finding of this work is that H_4B is not a stoichiometric component in the reaction catalyzed by NOS from rat cerebellum. Rather, H_4B plays a role in the prevention of progressive loss of enzyme activity. This conclusion is based on the following findings: (*i*) The initial rate of NOS is independent of H_4B. An effect of this factor is revealed only as incubation progresses (Fig. 1). (*ii*) The catalytic role of H_4B was demonstrated by the finding that at low concentrations each mole of added H_4B can support the formation of at least 15 mol of product (Fig. 2). (*iii*) The catalytic function of H_4B is not a result of its recycling. This was shown by direct demonstration that a purified preparation of NOS was unable to recycle detectable amounts of H_4B (Table 2). Further, NOS was not inhibited by concentrations of MTX that inhibit the enzymes that are most likely to be involved in recycling of H_4B (Table 3).

Our studies on the mechanism of action of H_4B are at a preliminary stage. Many of the effects of H_4B on NOS resemble, superficially at least, those of ascorbate in the prolyl hydroxylase reaction. In that reaction, ascorbate is not

a stoichiometric reactant and is required only after 15–30 reaction cycles, after which the reaction rate declines, probably due to oxidation of enzyme-bound iron in the catalytic process (32, 33). Catalytic turnover of enzyme is a necessary feature of this model. The finding that inactivation of NOS in the absence of H_4B proceeds without catalytic turnover (Table 4) argues against H_4B playing a role with NOS that is similar to that of ascorbate in the prolyl hydroxylase system.

Two viable models remain for the role of H_4B. In the first, H_4B maintains some group(s) on the enzyme in a reduced state required for activity. A precedent for this model is the role of H_4B in maintaining PAH in the reduced form required for enzyme activity; this requirement for H_4B is observed in the absence of catalytic turnover of enzyme (34). In the second model, H_4B acts as an allosteric effector to maintain the enzyme in an active form. Either of these models or a combination of them may be invoked. Our further finding that H_2B has a small but reproducible activity appears to be more compatible with the allosteric model. The small but significant MTX-insensitive stimulation by H_2B (Tables 1 and 3), coupled with the finding that H_2B is not recycled to H_4B, suggests that H_2B *per se* is capable of stimulating NOS. There is only a small likelihood that H_2B is acting as a reductant of NOS. Whereas 7,8-dihydropterins can reduce ferric iron, the rate of this reaction at pH 6.4 is at least 3 orders of magnitude less than the corresponding rate with tetrahydropterins (35). Further, H_2B typically acts as a biochemical oxidant (in the reaction catalyzed by DHFR), and no biological reaction is known in which H_2B acts as a reductant. The finding that the combined effect of H_2B and (6*R*)-H_4B is intermediate between that of either alone (Table 3) is also consistent with an allosteric model in which the two pterins compete for the same binding site.

The present evidence that H_4B is not a stoichiometric reductant in the transformation of arginine to nitric oxide and citrulline is not in accord with recent formulations that H_4B is a stoichiometric reductant and is regenerated in the NOS system (7, 12, 13). Stuehr *et al.* (13), for example, have implied that in the conversion of hydroxyarginine, the intermediate in the reaction, to the products, not only is 0.5 mol of NADPH oxidized per mol of citrulline formed, but also H_4B is stoichiometrically oxidized. An analysis of the net electron change involved in this reaction, however, indicates that there is no need to invoke any oxidation of H_4B. Therefore, none of the published data (7, 12, 13) are incompatible with either our results or our conclusion.

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