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BIOSYNTHESIS OF TETRAHYDROBIOPTERIN: CONVERSION OF DIHYDRONEOPTERIN TRIPHOSPHATE TO TETRAHYDROPTERIN INTERMEDIATES

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It is known that the first step in the de novo synthesis of tetrahydrobiopterin from GTP is the conversion of GTP to dihydroneopterin triphosphate. Recent evidence supports the conclusion that beyond this first step, the pterin intermediates in the pathway are all at the tetrahydro level of reduction. We have now shown that partially purified fractions from rat liver, rat brain and bovine adrenal medulla catalyze the conversion of dihydroneopterin triphosphate to tetrahydrobiopterin, as well as to the putative intermediates in the pathway, 6-pyruvoyl-tetrahydropterin and 6-lactoyl-tetrahydropterin. Results of both enzymatic and chemical studies support the assigned structures for the latter two tetrahydropterins. We have also purified extensively from brain an enzyme, distinct from sepiapterin reductase, that catalyzes the TPNH-dependent reduction of 6-pyruvoyl-tetrahydropterin to 6-lactoyl-tetrahydropterin. The role of this reductase in tetrahydrobiopterin synthesis has not yet been established.

The discovery that methotrexate, a potent inhibitor of dihydrofolate reductase (DHFR), had no effect on de novo synthesis of tetrahydrobiopterin (BH_4)(1) and the demonstration that neither sepiapterin (2) nor dihydrobiopterin (3) were, in fact, intermediates led to the suggestion that BH_4 biosynthesis might proceed through tetrahydropterin intermediates (3,4). Furthermore, the finding that tetrahydrosepiapterin (6-lactoyl-5,6,7,8-tetrahydropterin) could be converted to BH_4 by sepiapterin reductase, led us to postulate the scheme for the de novo synthesis of BH_4 shown in Figure 1 (3). Subsequently, several other groups also proposed that BH_4 synthesis proceeds through tetrahydropterin intermediates (5,6).

Recently, Smith and Nichol, using bovine adrenal medulla preparations (7), and Switchenko, et al, using Drosophila (5), reported that they were not able to detect the formation of 6-lactoyl-tetrahydropterin from dihydroneopterin

Abbreviations: BH_4, tetrahydrobiopterin; NH_2TP, 7,8-dihydroneopterin triphosphate; 6-pyruvoyl-PtH_4, 6-[1,2-dioxopropyl] tetrahydropterin; 6-lactoyl-PtH_4, 6-[2-hydroxy-1-oxopropyl] tetrahydropterin.

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triphosphate (NH_2TP), although both groups were able to demonstrate the formation of a different tetrahydropterin intermediate whose structure was not determined.

We have now studied the conversion of NH_2TP to BH_4 in preparations from rat liver and brain, and from bovine adrenal medulla. In contrast to the results of Smith and Nichol (7), we find that enzymes in all three tissues can catalyze the formation of three tetrahydropterins from NH_2TP which we have characterized as 6-pyruvoyl-, 6-lactoyl-, and 6-dihydroxypropyl-tetrahydropterin (BH_4).

Materials and Methods

Substrates and chemicals were as described previously (3). Preliminary studies on unfractionated extracts prepared from fresh rat liver and brain and frozen bovine adrenal medulla (Pel-Freeze) were carried out in order to be certain that the distribution of products formed from NH_2TP was not altered by the ammonium sulfate fractionation step described below. Tissues were homogenized (Polytron) in 4 volumes of 50 mM Tris-HCl, pH 7.4/1 mM phenylmethylsulfonyl fluoride/1 mM dithiothreitol/0.1 mM EDTA. After centrifugation at 40,000g for 1 hour, the extracts were fractionated with ammonium sulfate. The fraction precipitating between 35 and 60% saturation was reconstituted in 1/10 volume of the original extract in extract buffer. The final preparations from all three sources had a protein concentration of 20 mg/ml, as determined by the method of Warburg and Christian (8). The activities, described below, were purified 2-3 fold with about 80% recovery. All procedures were carried out at 4°.

Analysis of tetrahydropterins by HPLC with electrochemical detection: The HPLC system consisted of: a Gilson model 302 pump; a Waters U6K manual injector; a BAS model LC-4A electrochemical detector (0.3 V); an SP 4270 recording integrator. The solvent was 0.1M KH_2PO_4/2 mM octanesulfonic acid/0.1 mM EDTA/5% methanol (adjusted to pH 2.5 with H_3PO_4), pumped at 1.5 ml/min through an Altex C-18 column (5 μ, 4.6 x 150 mm).

6-Pyruvoyl-PtH_4 synthase assays: a) HPLC: Assays contained (in 0.1 ml): 10 μmol Tris (pH 7.4); 4 μmol dithiothreitol; 0.2 μmol $MgSO_4$; 2.5 nmol NH_2TP; and 15 μmol KCl. Samples were incubated for 15 min at 37°. Reactions were terminated by the addition of 10 μl of 30% (w/v) TCA. After centrifugation for 2 min in a microfuge, samples were kept on ice until analyzed by HPLC. Since the intermediates formed from NH_2TP were fairly unstable even under these conditions (approximately 10-20% loss per hour), analyses were always done as soon as possible after reactions were terminated. b) Cytochrome C reduction: tetrahydropterins reduce cytochrome C non-enzymatically (9). The rate of conversion of NH_2TP to a tetrahydropterin can be followed by measurement of the rate of the NH_2TP-dependent appearance of reduced cytochrome C, spectrophotometrically at 550 nm. Assay mixtures were prepared as described for the HPLC assay with the addition of 0.1 μmol horse heart cytochrome C. Blank reactions contained everything but NH_2TP.

6-Pyruvoyl-PtH_4 reductase assays: Reactions were carried out as described for the synthase assay, with the addition of 0.02 μmol of N-acetyl-serotonin and 0.02 μmol of TPNH.

BH_4 Synthesis: HPLC assays were the same as described for the synthase assay with the addition of 0.02 μmol TPNH.

Pyruvic and Lactic acid determinations: Tetrahydropterin-containing reaction mixtures were acidified by the addition of an equal volume of 0.2M H_3PO_4. MnO_2 (10 mg) was added to oxidize the pterins (10). After 10 min at room temperature, the MnO_2 was removed by centrifugation. Under these conditions, 6-pyruvoyl-PtH_4 and 6-lactoyl-PtH_4 give rise to pterin and pyruvic and lactic acids, respectively (11). Pyruvic acid was determined by HPLC after conversion to a

fluorescent quinoxaline derivative as described by Keike and Keike (12). Lactic acid was determined in the same manner, after conversion to pyruvic acid with the use of lactic dehydrogenase and DPN as a kit supplied from Sigma Chemical Co.

Reductions with NaB^3H_4: Reaction mixtures, prepared as described above, were terminated by placing on ice. 10 µl of 0.1M NaB^3H_4 (New England Nuclear) in 0.5M $NaHCO_3$ were added. After 30 min on ice and 15 min at room temperature, the only tetrahydropterin detected was BH_4 (the threo and erythro isomers of BH_4 are not separated). Samples were then acidified with H_3PO_4, oxidized with MnO_2 (10) and analyzed by HPLC on a C-18 column as previously described (13). Fractions were collected and the tritium incorporation determined. As expected, a 1:1 mixture of threo- and erythro-biopterin was formed from 6-pyruvoyl-PtH_4. Both isomers contained 2 mols of tritium per mol of pterin, confirming the structure of the diketo intermediate. Reaction mixtures containing 6-lactoyl-PtH_4, on the other hand, gave 1.3:1 mixtures of the threo and erythro isomers, with only 1 mol of ^3H incorporated in each isomer. There was no incorporation of ^3H into BH_4 itself.

Purification of 6-lactoyl-PtH_4 synthase from rat brain: Details of the purification will be presented elsewhere (S. Milstien and S. Kaufman, manuscript in preparation). Synthase activity was purified 12,500 fold with an overall recovery of 22% by the following steps: ammonium sulfate fractionation; heating at 80°; DEAE-Sephacel ion exchange chromatography; FPLC-mono Q anion exchange chromatography (Pharmacia); and high performance gel filtration.

Results and Discussion

In our proposed pathway for BH_4 biosynthesis (Fig. 1), the first product formed from NH_2TP is a tetrahydropterin. When extracts prepared from rat brain were incubated for short times with NH_2TP in the absence of any added reduced pyridine nucleotides or other electron donors, we detected the formation of a tetrahydropterin. This tetrahydropterin had the properties expected for the proposed diketo-tetrahydropterin: it was oxidized electrochemically at a low potential; upon chemical oxidation, it broke down to give pterin and pyruvic acid; it contained no phosphate as shown by the lack of effect of treatment with alkaline phosphatase on chromatographic properties (data not shown); reduction with NaB^3H_4 resulted in the formation of a mixture of the erythro and threo isomers of BH_4 (isomers identified after oxidation to biopterin) with 2 mols of tritium incorporated per mol of pterin in each isomer; and it was converted to BH_4 after reduction with TPNH, a reaction catalyzed by sepiapterin reductase (present results). The possibility that sepiapterin reductase could catalyze this reaction was suggested by the finding that it has diketo reductase activity (14). When TPNH and N-acetyl serotonin, the latter compound being a potent sepiapterin reductase inhibitor (15), were added to reactions which produced 6-pyruvoyl-PtH_4, it disappeared and 6-lactoyl-PtH_4 was formed. This product had identical chromatographic mobility and chemical properties to those of the compound

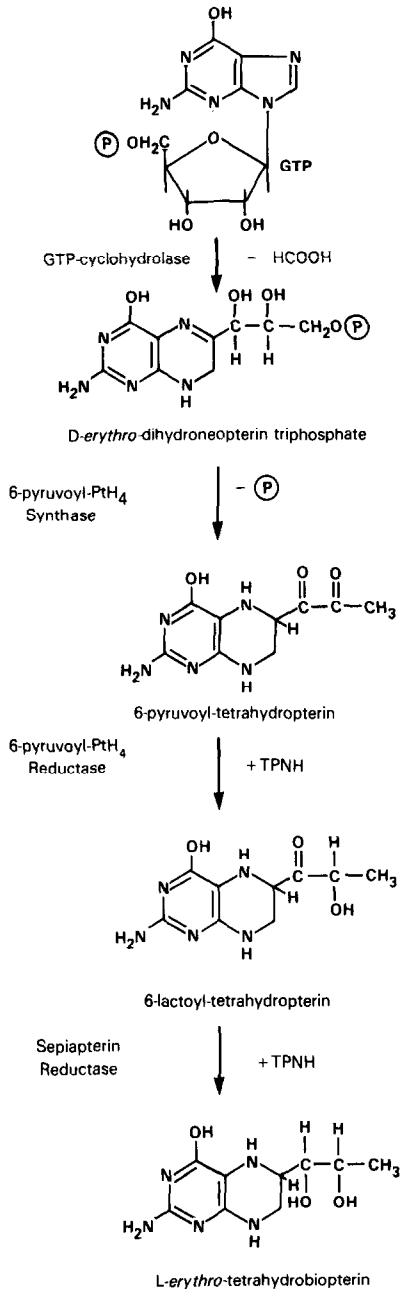


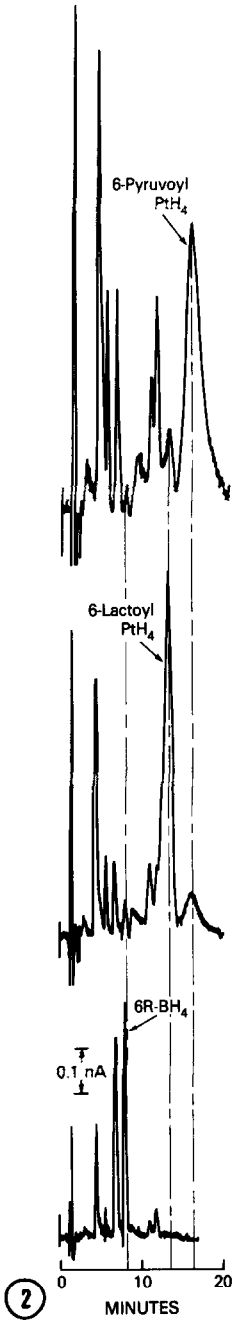
Figure 1. Proposed pathway for the De Novo Biosynthesis of Tetrahydrobiopterin.

produced by the TPNH-dependent reduction of 6-lactoyl-dihydropterin, catalyzed by DHFR (3). When reaction mixtures containing either of these tetrahydropterins were rapidly ultrafiltered (Amicon, MPS) to remove all proteins, and the ultrafiltrates then incubated with TPNH and pure sepiapterin reductase, both intermediates were converted to BH_4.

Smith and Nichol (4), using HPLC with electrochemical detection, were not able to detect the conversion of NH_2TP to 6-lactoyl-PtH_4 in bovine adrenal medulla preparations. Using their HPLC conditions, we found that both 6-pyruvoyl-PtH_4 and 6-lactoyl-PtH_4 had the same retention time, even though authentic 6-lactoyl-PtH_4 (produced by reduction with DHFR) had a different retention time then the 6-pyruvoyl-PtH_4 produced by incubations with tissue extracts. Mixtures of the two were not separated, indicating that some component(s) of the assay was interfering with the chromatography. However, as shown in Figure 2, with the use of both a different solvent and a different column, we were able to demonstrate the formation of 6-pyruvoyl-PtH_4 and 6-lactoyl-PtH_4 in rat brain extracts by HPLC with electrochemical detection. In Fig. 3 is shown a plot of peak heights of the three tetrahydropterins as a function of the applied potential. All three are oxidized at a half-maximal potential of about 0.2V. This value is characteristic of tetrahydropterins (5, 16). In addition, both rat liver and bovine adrenal medulla preparations were also shown to produce the same products from NH_2TP (see Table I).

In Table I are presented the results of the measurement of the rates of formation of 6-pyruvoyl-PtH_4, 6-lactoyl-PtH_4, and BH_4 from NH_2TP by ammonium sulfate fractionated extracts of rat brain, rat liver, and bovine adrenal medulla. As can be seen, all three tissues are capable of catalyzing the synthesis of these three tetrahydropterins. However, rat brain has a substantially higher ratio of reductase to synthase activity than do the other two tissues. This could account for some of the difficulty in isolation of 6-lactoyl-PtH_4 from bovine adrenal medulla (7).

The overall rate of formation of BH_4 from NH_2TP in all three tissues is greater than the synthase or reductase rates. Since the rate of formation of the final product in a pathway cannot be higher then the rates of formation of the intermediates, it is likely that the synthase and reductase rates are underestimated due to the instability of the lactoyl and pyruvoyl tetrahydropterins (5). Incubation of ultrafiltrates from synthase and reductase reaction mixtures at 37^{o} show that both of these pterins disappear with a half-life of about 5 and 10 minutes,



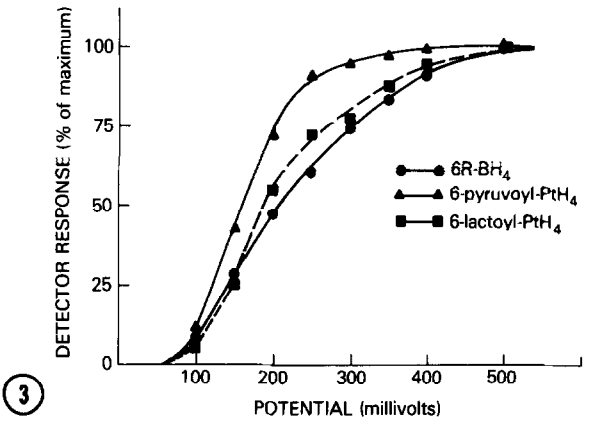


Figure 2. HPLC Assays of the rates of conversion of NH_2TP to 6-pyruvoyl-PtH_4, and BH_4 by rat brain extracts. Assays were carried out as described in Materials and Methods. Three reaction mixtures were incubated at 37° for 15 min, the reactions stopped with trichloroacetic acid and an aliquot of protein-free supernatant injected into the HPLC column. Only the labelled peaks were tetrahydropterins as judged by measurement of oxidation potentials and from results of additional experiments that were described in the text.

Figure 3. Voltagram of 6-pyruvoyl-PtH_4, 6-lactoyl-PtH_4 and BH_4. A mixture was made of protein-free supernatants from assays of the conversion of NH_2TP to the tetrahydropterins as described in Materials and Methods, catalyzed by rat brain extracts. The applied potential was varied and detector response (peak heights) was determined.

Table I. The Conversion of Dihydroneopterin Triphosphate to Tetrahydropterins

	6-Pyruvoyl-PtH_4 Synthase*	6-Pyruvoyl-PtH_4 Reductase*	BH_4 Synthesis*
Rat Brain	10.1	51.4	53.4
Rat Liver	21.0	21.0	126
Bovine Adrenal Medulla	27.6	15.9	73.7

*pmol/min/mg protein.
These rates were determined with the use of 35-60% ammonium sulfate fractions.
Products were measured as described in Materials and Methods.
Assays were linear with time to 15 min and with protein from, 0.2 to 1.0 mg per assay.

respectively. Assays were usually run in the presence of 40 mM dithiothreitol (DTT) to protect the tetrahydropterins from oxidation. Preliminary experiments showed that this amount of DTT gave 80-90% of the maximal amounts of products made in the presence of 100 mM dithiothreitol. Smith and Nicol (7) used only 5 mM DTT in their assays of bovine adrenal medulla activity. This amount of DTT in our assays gives only about 25% of the maximal amounts of 6-pyruvoyl-PtH_4 and also results in the formation of another tetrahydropterin which we have not identified. Since formation of this tetrahydropterin decreases with increasing DTT concentration, and since it also appears to be converted to BH_4 after the addition of sepiapterin reductase and TPNH, it could be the enol tautomer of the diketo tetrahydropterin.

In Table 2 are presented results of the measurement of the activity of purified brain 6-pyruvoyl-PtH_4 synthase by several different methods. There is substantially more product formed, as determined by measurement of the total amounts of pyruvic acid and pterin formed, than is determined by measurement of the rate of appearance of 6-pyruvoyl-PtH_4 by HPLC or by cytochrome C reduction. These results are in agreement with the known lack of stability of this pterin towards oxidation (5).

The question of whether 6-lactoyl-PtH_4 is an intermediate in the de novo BH_4 biosynthetic pathway remains to be answered. It appears that sepiapterin reductase, an enzyme previously thought to catalyze only the reduction of sepiapterin to 7,8-dihydrobiopterin (17), is required for BH_4 biosynthesis (3).

Table 2. Measurement of Rat Brain 6-Pyruvoyl-PtH_4 Synthase Activity

Assay Method	pmol/15 min
HPLC-ECD	1822
Cytochrome C Reduction	2085
Pyruvic Acid Formation	3312
Pterin Formation	3200

A preparation of rat brain 6-pyruvoyl-PtH_4 synthase, purified through the FPLC step (36 μg/assay, specific activity, 2400 pmol/min/mg), was used for all of the assays performed as described in the text.

Sepiapterin reductase catalyzes the reduction of both 6-pyruvoyl-PtH_4 and 6-lactoyl-PtH_4 to BH_4. There is no detectable 6-lactoyl-PtH_4 intermediate in the reduction of the 6-pyruvoyl-PtH_4 by purified sepiapterin reductase. Inhibition of sepiapterin reductase completely blocks the de novo formation of BH_4. However, as documented above, in the presence of an inhibitor of sepiapterin reductase and TPNH, 6-pyruvoyl-PtH_4 is converted at relatively high rates to 6-lactoyl-PtH_4. With the use of purified 6-pyruvoyl-PtH_4 synthase, we have been able to isolate from brain a TPNH-dependent reductase, distinct from sepiapterin reductase, that catalyzes the reduction of 6-pyruvoyl-PtH_4 to 6-lactoyl-PtH_4 (S. Milstien and S. Kaufman, in preparation). Addition of 6-lactoyl-PtH_4 to complete reactions for de novo synthesis of ^{14}C-BH_4 from ^{14}C-NH_2TP results in inhibition of the formation of ^{14}C-BH_4. Although it is likely that this inhibition is due to trapping of radioactivity by the added 6-lactoyl-PtH_4, a result that would support the idea that 6-lactoyl-PtH_4 is an intermediate in the conversion of NH_2TP to BH_4, we have not, because of the instability of these tetrahydropterins, been able to isolate ^{14}C-6-lactoyl-PtH_4.

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