Pterin and Folate Salvage. Plants and *Escherichia coli* Lack Capacity to Reduce Oxidized Pterins

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Dihydropterins are intermediates of folate synthesis and products of folate breakdown that are readily oxidized to their aromatic forms. In trypanosomatid parasites, reduction of such oxidized pterins is crucial for pterin and folate salvage. We therefore sought evidence for this reaction in plants. Three lines of evidence indicated its absence. First, when pterin-6-aldehyde or 6-hydroxymethylpterin was supplied to Arabidopsis (*Arabidopsis thaliana*), pea (*Pisum sativum*), or tomato (*Lycopersicon esculentum*) tissues, no reduction of the pterin ring was seen after 15 h, although reduction and oxidation of the side chain of pterin-6-aldehyde were readily detected. Second, no label was incorporated into folates when 6-[3H]hydroxymethylpterin was fed to cultured Arabidopsis plantlets for 7 d, whereas [3H]folate synthesis from p-[3H]aminobenzoate was extensive. Third, no NAD(P)H-dependent pterin ring reduction was found in tissue extracts. Genetic evidence showed a similar situation in *Escherichia coli*: a GTP cyclohydrolase I (folE) mutant, deficient in pterin synthesis, was rescued by dihydropterins but not by the corresponding oxidized forms. Expression of a trypanosomatid pterin reductase (PTR1) enabled rescue of the mutant by oxidized pterins, establishing that *E. coli* can take up oxidized pterins but cannot reduce them. Similarly, a GTP cyclohydrolase I (fol2) mutant of yeast (*Saccharomyces cerevisiae*) was rescued by dihydropterins but not by most oxidized pterins, 6-hydroxymethylpterin being an exception. These results show that the capacity to reduce oxidized pterins is not ubiquitous in folate-synthesizing organisms. If it is lacking, folate precursors or breakdown products that become oxidized will permanently exit the metabolically active pterin pool.

Pterins have a wide range of metabolic roles, including as essential intermediates in folate biosynthesis, as products of folate breakdown (Scott et al., 2000; Suh et al., 1991), and as cofactors for aromatic hydroxylases (Zhao et al., 1994; Thony et al., 2000; Yamamoto et al., 2001). The oxidation state of the pterin ring (Fig. 1A) is crucial to biochemical function because only reduced (dihydro or tetrahydro) forms are metabolically active. The intermediates of folate synthesis are dihydro forms, as is the major pterin formed by folate breakdown (Fig. 1B). Pterin cofactors are tetrahydro forms.

Reduced pterins readily autoxidize to their fully oxidized (aromatic) state (Fig. 1B, mauve arrows; Pfleiderer, 1985). A capacity to reduce oxidized pterins to their bioactive forms would thus seem a likely auxiliary to folate and pterin pathways. So far, however, only Leishmania and other trypanosomatid parasites are definitively known to have this capacity. These organisms, which are pterin and folate auxotrophs, contain an NADPH-dependent pterin reductase (PTR1, EC 1.5.1.33) that mediates the two-stage reduction of pterins to their 7,8-dihydro and tetrahydro states (Bello et al., 1994; Dawson et al., 2006). PTR1 is a member of the short chain dehydrogenase/reductase (SDR) family with a distinctive variant of the usual SDR motif (TGX3GXG, where X is any amino acid) in which R replaces the second G (Gourley et al., 2001). PTR1 is distinct from 6,7-dihydropterin reductase (EC 1.5.1.34, also termed quinonoid dihydropteridine reductase), which acts on 6,7-dihydropterins. These pterins are intermediates in the recycling of tetrahydropterin cofactors (Thony et al., 2000).

It is not clear whether folate-synthesizing organisms (plants, bacteria, and fungi) have a reductase that acts on oxidized pterins. Some evidence suggests not. Thus, in tomato (*Lycopersicon esculentum*) fruit with up-regulated pterin synthesis, most of the accumulated pterins became oxidized as ripening proceeded (Diaz de la Garza et al., 2004). In *Lactobacillus plantarum*, 6-hydroxymethylpterin (PtCH2OH) and pterin-6-aldehyde (PtCHO) were far poorer folate synthesis precursors in cell extracts than their reduced forms (Shiota, 1959). Similarly, in *Escherichia coli*, neither PtCH2OH (Shiota and Disraely, 1961) nor pteroic acid (Griffin and Brown, 1964) supported in vitro folate synthesis, although their dihydro forms did. Furthermore, the only PTR1-like enzyme in *E. coli*, FolM, acts on dihydrobiopterin but not on its oxidized form (Giladi et al., 2003).

While it is unclear whether bacteria or plants reduce oxidized pterins to the dihydro level, it is certain that bacteria reduce dihydropterin to the tetrahydro level,
and likely that plants do. Thus, tetrahydromonapterin and tetrahydrohydroxymethylpterin (the latter as a glycoside) occur in bacteria, both being reduction products of folate synthesis intermediates (Fig. 1B; Guroff and Rhoads, 1969; Lee et al., 1999; Ikemoto et al., 2002). Furthermore, the tetrahydropterin-dependent enzyme Phe hydroxylase occurs naturally in Pseudomonas and some other bacteria, and functions if introduced into E. coli (Zhao et al., 1994). Lastly, plant genomes encode homologs of mammalian pterin 4a-carbinolamine dehydratase, an enzyme needed to recycle tetrahydropterin cofactors (Thony et al., 2000) that may be considered diagnostic for their presence.

The reduction of oxidized pterins could impact the availability of folate synthesis intermediates and the salvage of folate breakdown products. It is also relevant to biofortification projects in which pterin synthesis is up-regulated to enhance folate accumulation (Sybesma et al., 2003; Diaz de la Garza et al., 2004; Hossain et al., 2004) since overproduced pterins, which can autoxidize, are at best useless and may even inhibit folate synthesis (Brown et al., 1961). We therefore investigated pterin reduction in plants, focusing particularly on the possible reduction of PtCH₂OH, the oxidized form of the folate synthesis intermediate 6-hydroxymethyl-dihydropterin (H₂PtCH₂OH), and PtCHO, a folate breakdown product (Fig. 1B) that is convertible to PtCH₂OH by side chain reduction (Kobayashi, 1982; Orsomando et al., 2006). Having found no reductive capacity in plants, we checked the generality of this surprising result in E. coli and yeast (Saccharomyces cerevisiae).

RESULTS

Absence of PTR1-Like Plant Proteins

Because trypanosomatids acquired many genes from photosynthetic organisms (Hannaert et al., 2003), we searched plant genomes for SDR sequences resembling Leishmania PTR1 in having a TGX₃RXG motif. Among 86 SDRs in the Arabidopsis (Arabidopsis thaliana) genome and similar numbers in rice (Oryza sativa) and poplar (Populus spp.) genomes, none had this motif. Nor did any of them have higher overall sequence identity to PTR1 than the 15% to 30% typical for SDR family members (Jörnvall et al., 1999). Plants thus do not have PTR1 orthologs, suggesting possible inability to reduce oxidized pterins.

Absence of in Vivo Pterin Reduction in Plants

When Arabidopsis, pea (Pisum sativum), and tomato tissues were incubated with PtCH₂OH it was readily absorbed and in most cases underwent some side chain oxidation to pterin-6-carboxylate (PtCOOH; Fig. 2, A and B). Neither the remaining PtCH₂OH nor the PtCOOH showed measurable ring reduction (Fig. 2C). The absence of detectable reduced PtCH₂OH (di- or tetrahydro, henceforth for simplicity H₂PtCH₂OH), cannot be ascribed to in vivo decomposition or metabolism since it accumulated substantially when dihydropterin-6-aldehyde (H₂PtCHO) replaced PtCH₂OH (Fig. 1B); H₂PtCH₂OH in this case comes from side chain reduction (Orsomando et al., 2006). These data imply that plants lack PtCH₂OH and PtCOOH ring reductase activity.

To test for ring reductase activity against PtCHO, we reasoned that the product, H₂PtCHO, would undergo
rapid side chain reduction to give H$_2$PtCH$_2$OH, as occurs with exogenously supplied H$_2$PtCHO (Fig. 2C). As expected (Orsomando et al., 2006), Arabidopsis, pea, and tomato tissues all reduced the PtCHO side chain to give PtCH$_2$OH, but none of this was in reduced form (Fig. 3). Consistent with the observed conversion of PtCH$_2$OH to PtCOOH (Fig. 2A), all tissues also metabolized PtCHO to PtCOOH, none of which was reduced. These results indicate that PtCHO ring reductase activity is lacking, and corroborate the results of Figure 2C for PtCH$_2$OH and PtCOOH.


The above results were obtained with quite short incubation times (15 h) and with pterin doses (approximately 20 nmol g$^{-1}$ fresh weight) that exceeded endogenous pterin contents (e.g., Fig. 2B). We therefore carried out longer experiments with a more physiological pterin dose, exploiting the fact that pterins need reduction to the dihydro level before incorporation into folates (Fig. 1B). Axenically cultured Arabidopsis plantlets (initial fresh weight 0.25 g) were given 166 pmol (1.9 μCi) of $[^7]$H PtCH$_2$OH for 7 d, then analyzed for $[^3]$H incorporation into folates. Control plantlets received a similar dose of $[^7]$H aminobenzoate ($[^7]$H PABA; 65 pmol, 1.7 μCi), PABA being readily incorporated into folates by plants (Imeson et al., 1990). At 7 d, similar amounts of label from $[^7]$H PtCH$_2$OH and $[^7]$H PABA were present in tissue extracts (0.74 and 1.15 μCi, respectively), demonstrating comparable uptake. However, while folates were heavily labeled in $[^7]$H PABA-fed samples they contained no detectable label in $[^7]$H PtCH$_2$OH-fed samples (Fig. 4). In the latter, 98% of the $[^3]$H in the extract was in the form of PtCOOH, consistent with the extensive PtCH$_2$OH $\rightarrow$ PtCOOH conversion in Figure 2A, and 2% remained in PtCH$_2$OH. This lack of conversion of $[^7]$H PtCH$_2$OH to folates implies that it was not reduced to its bioactive dihydro form. It is unlikely that the lack of folate labeling from $[^7]$H PtCH$_2$OH was due to loss of $[^3]$H by exchange with...
water either before or after incorporation into folates. Tritium or deuterium at position 7 of the pterin ring is subject to very little if any spontaneous exchange, or to exchange during enzymatic reduction and tetra- to dihydro oxidation (Kaufman, 1964; Zakrzewski, 1966; Scott, 1984; Charlton et al., 1985). Experimental evidence against exchange as an explanation for lack of folate labeling is that only 3% of the [3H]PtCH2OH label supplied was recovered from the medium as 3H2O at the end of the experiment. Nor can the absence of [3H]folate synthesis from [3H]PtCH2OH be explained by massive isotopic dilution by the endogenous PtCH2OH pool, because this pool is far smaller in Arabidopsis than the total PABA pool (0.2 versus approximately 5 nmol g\(^{-1}\) fresh weight; Fig. 3; Orsomando et al., 2006).

**Absence of Pterin Reduction by Plant Extracts**

We tested for PtCHO or PtCH2OH ring reductase activity simultaneously using a coupled assay that exploits the high NADPH-linked pterin aldehyde reductase activity of plant extracts (Orsomando et al., 2006). PtCHO was used as substrate; the predicted end product was H2PtCH2OH, the pathway to it being PtCHO \(\rightarrow\) H2PtCHO \(\rightarrow\) H2PtCH2OH for PtCHO ring reductase, and PtCHO \(\rightarrow\) PtCH2OH \(\rightarrow\) H2PtCH2OH for PtCH2OH reductase. In the presence of NADPH and an NAD(P)H regenerating system, Arabidopsis, pea, and tomato extracts converted PtCHO to PtCH2OH, but none of this was reduced (Fig. 5). Tests with PtCH2OH as substrate likewise gave no NADPH- or NADH-dependent H2PtCH2OH formation (data not shown).

In view of the oxidation of PtCHO to PtCOOH seen in vivo (Fig. 3), we also tested for pyridine

**Figure 3.** Metabolism of PtCHO by plant tissues. Arabidopsis leaf, pea leaf, and tomato pericarp tissues (0.15–0.23 g fresh weight) were incubated with PtCHO (4 nmol) for 15 h in darkness and washed. Pterins were analyzed before (−Ox) and after (+Ox) oxidation. Top frames show formation of PtCH2OH and PtCOOH (means of three replicates and st). Values were corrected for the low endogenous levels of these pterins in controls incubated without PtCHO, shown for representative nonoxidized samples in the bottom frames.

**Figure 4.** Metabolism of 6-[3H]hydroxymethylpterin and [3H]PABA by Arabidopsis plantlets. Plantlets (initial fresh weight 0.25 g) in 100 mL of medium were supplied with 1.6 μCi (166 pmol) of [3H]PtCH2OH or 1.7 μCi (65 pmol) of [3H]PABA and harvested after 7 d. Tissue fresh weight at harvest was 4.9 g. The folate fraction was deglutamylated, isolated by affinity chromatography, and separated by HPLC. Running positions of authentic folates cochromatographed with the samples are indicated. The experiment was repeated, with similar results. THF, Tetrahydrofolate; 5-CH3-THF, 5-methyltetrahydrofolate; 5,10-CH5THF, 5,10-methenyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; DHF, dihydrofolate; FA, folic acid. The radioactive peaks at approximately 17 and 27 min are unidentified metabolites.
nucleotide-dependent and -independent PtCHO oxidation (Fig. 5). Arabidopsis, pea, and tomato extracts had substantial NAD-linked activity, and pea and tomato also had considerable activity without added NAD or NADP. The latter activity was not due to traces of endogenous NAD(P) left after desalting as it persisted when these were removed by an NAD(P)H regenerating system.

Folate Synthesis from Dihydropterins But Not Oxidized Pterins in *E. coli*

The above evidence for plants prompted us to investigate whether other folate-synthesizing organisms, *E. coli* and yeast, also lack pterin-reducing capacity. To find whether *E. coli* has pterin reductase activity, we used a *folE* deletant (Klaus et al., 2005) that lacks GTP cyclohydrolase I (the first enzyme of pterin synthesis) and therefore cannot make pterins or folates (Fig. 1B). This deletant can be maintained on Luria-Bertani (LB) medium plus thymidine, but its growth is slow (Fig. 6A, top row). Various oxidized pterins and dihydropterins were tested for the ability to support growth (Fig. 6A); they were related either to folate synthesis (PtCH$_2$OH, neopterin, monapterin, pteroic acid, and their dihydro forms) or to folate breakdown (PtCHO and H$_2$PtCHO; Fig. 1B). All the dihydropterins allowed rapid growth of the deletant, comparable to that of wild-type cells (Fig. 6A, first column). That H$_2$PtCHO was as effective as H$_2$PtCH$_2$OH confirms the in vitro evidence (Mitsuda and Suzuki, 1971) that *E. coli* can reduce the H$_2$PtCHO side chain. Unlike the dihydro forms, oxidized pterins did not support growth of the deletant (Fig. 6A, second column). This was not due to toxicity of oxidized pterins, since these did not affect the growth of wild-type cells (Fig. 6A, third column). Nor was it due to failure to take up oxidized pterins, since deletant cells expressing *Leishmania* PTR1 were able to utilize PtCH$_2$OH or PtCHO (Fig. 6B), both of which are PTR1 substrates (Nare et al., 1997). The failure of the *folE* deletant to grow on oxidized pterins thus argues strongly that, like plants, *E. coli* lacks capacity to reduce them.

DISCUSSION

We report here several lines of evidence that plants and *E. coli* have no detectable capacity to reduce the...
ring of oxidized pterins, although oxidation or reduction of the side chain is easily measurable. For plants, the metabolic, radiotracer, and biochemical lines of evidence are individually criticizable, on the grounds of abnormal compartmentation of pterins fed in vivo, for example. Collectively, however, these strands of evidence are quite persuasive, and made more so by the unequivocal genetic evidence for a similar situation in \textit{E. coli}. We therefore infer that, unlike trypanosomatids, plants and \textit{E. coli} have very little if any potential to salvage oxidized pterins, and that if this potential exists at all, it is physiologically insignificant compared to the capacity to modify the side chain.

This seems surprising, given the instability to oxidation of di- and tetrahydropterins, for it implies (1) that the dihydropterin intermediates of folate synthesis (H$_2$PtCH$_2$OH, dihydroneopterin, and dihydromonapterin) cannot be reclaimed if they become oxidized, and (2) that the folate breakdown product H$_2$PtCHO can only be recycled to folate synthesis if its side chain is reduced before its ring gets oxidized. Mechanisms can, however, be envisioned that would obviate the need to reduce oxidized pterins. The dihydropterin intermediates of folate synthesis could well be largely protein bound, and in this state resist oxidation. This is the case for tetrahydrofolates in mammals (Suh et al., 2001; Jones and Nixon, 2002). For the breakdown product H$_2$PtCHO, a sufficiently high side chain reductase activity could intervene to reduce it to H$_2$PtCH$_2$OH almost as it formed, preempting the chance of ring oxidation. 

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Pterin utilization by an \textit{E. coli} pterin synthesis mutant. A, Cells of four independent clones of the $\Delta$folE deletant ($\Delta$folE) were streaked on LB medium containing 0.1% Na ascorbate, 1 mM dithiothreitol, and kanamycin (50 $\mu$g mL$^{-1}$), without or with 300 $\mu$M thymidine (dT) or 11 $\mu$M pterin (dihydro or oxidized). The inoculum was from a thymidine-containing plate. Incubation was at 37$^\circ$C under N$_2$. Conditions were the same for the K12 wild-type (WT) strain except that kanamycin was omitted and the inoculum was from an LB plate. Plates were photographed after 1 d. B, folE Deletant cells were transformed with pBluescript alone (V) or harboring \textit{Leishmania major} PTR1 (PTR1). Six independent clones of each construct were streaked on LB medium containing 0.5 mM isopropyl-\textbeta\text-d-thiogalactopyranoside, kanamycin (30 $\mu$g mL$^{-1}$), and ampicillin (60 $\mu$g mL$^{-1}$) without or with 300 $\mu$M thymidine or 11 $\mu$M pterin. Plates were incubated at 37$^\circ$C under N$_2$ and photographed after 5 d. NPt, Neopterin; MPt, monapterin; Pte, pteroate. The experiment was repeated, with similar results.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure7.png}
\caption{Pterin utilization by a yeast pterin synthesis mutant. Cells of four independent clones of the $\Delta$fol2 mutant and the corresponding wild type (WT) were streaked twice in succession on YPD medium containing 0.2% Na ascorbate and 1 mM dithiothreitol, without or with 100 $\mu$M folinic acid (FA) or 20 $\mu$M pterin (dihydro or oxidized). Plates were incubated at 30$^\circ$C under N$_2$, and photographed after 2 d. Pterin abbreviations as in Figure 6. The experiment was repeated, with similar results.}
\end{figure}
oxygenation and PtCHO activity were determined in extracts of Arabidopsis leaves, pea leaves, and tomato pericarp. The specific activity of PtCHO was calculated using the HPLC conditions given below. Overall radiochemical yield was approximately 30% and radiochemical purity was ≥94%. The specific activities of product batches were 9 to 11 Ci mmol⁻¹.

**Plant Materials**

Arabidopsis L. Heynh. (ecotype Columbia) leaves were from rosettes of plants grown in a chamber for 4 to 6 weeks at 23°C to 28°C in 12-h d (photosynthetic photon flux density 80 μE m⁻² s⁻¹) in potting soil. Pea (Pisum sativum L. cv Laxon’s Progress 9) leaves were from 9- to 14-old plants grown as described (Orsomando et al., 2006). Tomato (Lycopersicon esculentum Mill.) pericarp was from mature green fruits of cv Micro-Tom, grown as described (Díaz de la Garza et al., 2004); tomato leaves were fully expanded, from a derivative of cv M82 growing in soil in a naturally lit greenhouse, in September, 2005.

**Escherichia coli and Yeast Strains**

Escherichia coli K12 foldl deletant P1-7B (Klaus et al., 2005) and strain P1-6E were obtained by the same procedure that were grown at 37°C on LB medium containing 300 μg/ml thymidine and 30 μg/ml kanamycin. Both strains were used in experiments. Yeast (Saccharomyces cerevisiae) strains 971/6 (Mata adz-1 his3-11,15 leu2-3,112 ura3-1 can1) and 971/6a (Mata adz-1 his3-11,15 leu2-3,112 ura3-1 can1 fol2; HIS3) were obtained from M. L. Agostoni Carbone (Università di Milano). Yeast was cultured at 30°C in YPD medium (1% Difco yeast extract, 1% Difco bacto-peptone, and 2% Glc). The fol2 mutant strain 971/6a was maintained on YPD containing formic acid (Ca²⁺ salt; Sigma) at a final concentration of 50 μg ml⁻¹.

**Pterin Metabolism Experiments**

Arabidopsis leaf sections and tomato pericarp discs were prepared as described (Orsomando et al., 2006). Pea and tomato leaf discs (1 cm diameter) or pea leaflets were stripped of the midvein on the abaxial surface. Pterin doses (19–32 μl of solution) were equally divided among sets of five or six leaf sections, leaflets, or discs, or two pericarp discs, applying droplets to the cut surfaces. Tissues were then incubated for 14.5 h in darkness on moist filter paper, shaken (75 rpm) for 0.5 h in 5 ml of water to remove nonabsorbed pterins, and frozen and ground in liquid N₂. Ground samples were extracted in 2 ml of MeOH:CHCl₃ (water (12:51), v/v) containing 0.1% (w/v) Na ascorbate and 10 mM β-mercaptoethanol, warmed to 50°C for 5 min, and centrifuged to clear. The supernatant was mixed with 0.5 ml of CHCl₃, and 0.75 ml of water and centrifuged to separate the aqueous phase, which was concentrated in vacuo to 200 μl. Pairs of 50-μl samples were taken for pterin analysis: one received 80 μl of 1 N HCl containing 1% L-proline 2% KI (w/v); to oxidize pterins, the other 80 μl of 1 N HCl alone. After incubation for 1 h in darkness, both samples received 10 μl of 5% (w/v) Na ascorbate (to destroy excess L-proline) and 7 μl of 1% NaOH (to partly neutralize the HCl). Aliquots (50 μl) were analyzed by HPLC. Recoveries from tissue samples of 200 pmol pterin spikes (used to correct experimental data) were: H₂PtCHO, 21% to 50%; PCH₂OH, 54% to 83%; and PCCOÖH, 8% and 78%. Recoveries from Arabidopsis were consistently lower than from pea or tomato. PCH₂OH and PCCOÖH recoveries from oxidized and nonoxidized samples were not significantly different.

**3H-Labeling Experiments**

Arabidopsis seeds were surface sterilized and germinated on Murashige and Skoog medium. Five-day-old seedlings were then transferred to a dark incubator (25°C, 0–2 lux; light:dark, 24:0 h) for 2 days, then kept in continuous light, and then exposed for 2 h to a 3H label (seven plantlets/fask) containing 100 μl of 0.333 μl liquid Murashige and Skoog medium plus 10 g L⁻¹ Suc. Flasks were shaken at 80 rpm; temperature and lighting were as given above for Arabidopsis. Filter-sterilized [7-3H]PtCH₂OH or [3,5-3H]PABA was added to 11-d-old cultures, which were darkened for the first 24 h, then returned to the normal light regime. At 7 d, plantlets were washed twice for a total of 4 h with 100 ml of culture medium containing 1 μl unlabeled PCH₂OH or PABA (to remove nonabsorbed label), then taken for analysis. Folates were extracted, deglutamylated, purified by affinity chromatography, and separated by HPLC as described (Díaz de la Garza et al., 2004).

**In Vitro Pterin Reduction and Oxidation Assays**

Extracts of Arabidopsis leaves, pea leaves, and tomato pericarp were prepared by grinding in liquid N₂, thawing in two volumes of 100 mM K

**Materials and Methods**

**Chemicals**

Pteridines were from Schircks Laboratories; near-saturated solutions were freshly prepared in N₂-purged K phosphate 2 mM, pH 8.5, and quantified spectrophotometrically using published extinction coefficients (Blakley, 1969; Pfleiderer, 1985; Orsomando et al., 2006). Pterin solutions were protected from light. [3,5-3H]Aminohippuric acid (26.2 Ci mmol⁻¹) and [3',5',7,9H]folic acid (47.9 Ci mmol⁻¹) diammonium salt in aqueous ethanol were from Morave Biochemicals.

**Synthesis of 6-[3H]-Hydroxymethylpterin**

[3H]Folic acid (48 μCi, 1 nmol) was dried in vacuo, redissolved in 100 μl of 50 mM K phosphate, pH 7.5, and irradiated for 230 s in a microcuvette in the UV beam of a Beckman DU 7400 diode array spectrophotometer. The [7-3H]PtCHO formed was enzymatically reduced to [7-3H]PtCH₂OH as follows. The volume was brought to 200 μl, and 10 mM glutathione, 100 mM NADPH, and 10% (v/v) glyceral (final concentrations) were added along with 20 μg purified recombinant aldehyde reductase (the Arabidopsis [Arabidopsis thaliana] At01g0330 gene product; A. Noirel and A. D. Hanson, unpublished data). After incubation for 1 h at 30°C, 6 μl of 10 N HCl were added to stop the reaction and to destroy excess NADPH, and after a further 1 h at 4°C denatured enzyme was removed by centrifugation (10,000g, 10 min). The [7-3H]PtCH₂OH product was isolated using the HPLC conditions given below. Overall radiochemical yield was approximately 30% and radiochemical purity was ≥94%. The specific activities of product batches were 9 to 11 Ci mmol⁻¹.
phosphate, pH 7.5, containing 5 mM diithiothreitol and 3% (w/v) polyvinylpyrrolidone, centrifuging to clear (10,000 × g, 10 min), and desalting on PD-10 columns equilibrated in 100 mM K phosphate, pH 7.5, containing 5 mM diithiothreitol and 10% (v/v) glycerol. The desalted extracts were frozen in liquid N₂ and stored at −80°C. Enzyme assays (50 μL final volume) contained 4 to 29 μg of protein, 100 mM K phosphate, pH 7.5, 10 mM glutathione, and 20 μM PECO. Reduction assays contained 200 μM NADPH and an NADPH-regenerating system comprising 1 mM Glc-6-P and 0.15 units (1 unit = 1 nmol min⁻¹) measured in the above assay buffer of Leuconostoc mesenteroides Glc-6-P dehydrogenase. Oxidation assays contained 200 μM NAD or NADP. Assays were incubated for 30 min at 30°C and stopped by freezing or acidification. A pair of 20-μL samples was used for pterin analysis: one received 10 μL of HCl-L/KI solution, the other 10 μL of 1 N HCl; both were incubated for 1 h in darkness and then received 10 μL of 5% (w/v) Na ascorbate and 60 μL of 10 mM Na phosphate, pH 6.0, containing 10 mM β-mercaptoethanol and 1% (w/v) Na ascorbate.

HPLC Analysis of Pterins

Pterins (50-μL injections) were separated on a 4-μm, 250-× 4.6-mm Synergi Fusion-RP 80 column (Phenomenex) eluted isocratically with 10 mM Na phosphate (pH 6.0) at 1.5 mL min⁻¹. Peaks were detected by fluorescence (350 nm excitation, 450 nm emission) and quantified relative to standards. Because pterins are highly fluorescent when oxidized but not when reduced, the difference in peak area between oxidized and nonoxidized samples is a measure of reduced (di- and tetrahydro) forms (Fukushima and Nixon, 1980).

Pterin Reduction Tests with E. coli and Yeast

E. coli cells were streaked on LB medium containing 0.1% (w/v) Na ascorbate, 1 mM diithiothreitol, 30 or 50 μM β-mercaptoethanol, and (for cells harboring pBluescript plasmids) 60 μM L-ascorbic acid, 1 mM dithiothreitol, 30 or 50 μM β-mercaptoethanol. Pterin concentration was 11 μM. Yeast cells were streaked on YPD medium containing 0.2% Na ascorbate and 60 μL of 10 mM Na phosphate, pH 6.0, containing 10 mM β-mercaptoethanol and 1% (w/v) Na ascorbate.

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LITERATURE CITED


Guroff G, Rhoads CA (1969) Phenylalanine hydroxylation by Pseudomonas species (ATCC 11299a); nature of the cofactor. J Biol Chem 244: 142–146


Mitsuda H, Suzuki Y (1971) Enzymatic conversion of 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine to 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine by cell-free extracts of Escherichia coli B. J Vitaminol (Kyoto) 17: 5–9


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