Nutrient Metabolism

Formyltetrahydrofolates Associated with Mitochondria Have Longer Polyglutamate Chains Than the Methyltetrahydrofolates Associated with Cytoplasm in Rat Brain

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ABSTRACT The subcellular distribution of folate coenzymes in the brain is unknown. Brain folate concentrations are low and hence require a sensitive assay to determine the subcellular distribution. Rat brain was fractionated by differential centrifugation into cytoplasmic, mitochondrial and crude synaptosomal fractions. The compositions of the folate pools in these subcellular fractions were determined by differential conversion of one-carbon forms enzymatically to 5,10-methylenetetrahydrofolate (5,10CH$_2$H$_4$PteGlun) followed by reaction of the 5,10CH$_2$H$_4$PteGlun with thymidylate synthetase and $[3$H]fluorodeoxyuridylate to form ternary complexes, which were then separated as a function of polyglutamate chain length by isoelectric focusing, visualized by fluorography and quantified by densitometry. The distribution of the pteridine derivatives in brain was very similar to the distribution of these derivatives in liver. Cytoplasm contained primarily 5-methyltetrahydropteroylglutamates with smaller amounts of unsubstituted tetrahydropteroyl-polyglutamates, whereas mitochondria contained approximately equal concentrations of unsubstituted and formyl-substituted tetrahydropteroylglutamates. The subcellular distribution of polyglutamate derivatives in brain, however, was different from that in liver. In the brain, the mitochondrial folates exhibited longer polyglutamate chains than did the cytoplasmic folates, a pattern opposite to that in the liver. Whereas the brain cytoplasmic pteroylglutamates were primarily hexa and hepta glutamates, the brain mitochondrial pteroylglutamates were primarily hexa and hepta glutamates. The brain also contained small but measurable levels of oxidized folates, which were seen in crude synaptosomal fractions but not in cytoplasmic or mitochondrial fractions. J. Nutr. 126: 3077–3082, 1996.

INDEXING KEY WORDS:
- rats
- brain
- mitochondria
- cytoplasm
- folate

Folate-dependent one-carbon metabolism is vital to all living cells. The one-carbon units generated in this pathway are essential for the synthesis of the purines and thymidylate and for the synthesis of the methyl group de novo. Biological folates exist in many different forms with differences either in the pteridine moiety or in the length of the poly-gamma-glutamate chain. The pteridine moiety can differ in the oxidation state of the B ring from folinic acid [(PteGlu)], in which positions 5, 6, 7 and 8 are fully unsaturated, to tetrahydrofolate (H$_2$PteGlun), in which these positions are fully saturated [i.e., reduced]. Although the unsaturated forms, PteGlun and dihydrofolic acid (H$_2$PteGlun), have not been shown to have any specific biological function, both can be reduced to the tetrahydro form in biological systems. In addition, the one-carbon units that are substituted at the 5 and 10 nitrogens can vary in oxidation state from the formate oxidation state of 5-formyl-

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2 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.
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4 Abbreviations used: 5,10CH$_2$H$_4$PteGlun, 5,10-methylenetetrahydropteroyl-n-glutamate; 5,10CH$_2$H$_4$PteGlun, 5,10-methylenetetrahydropteroyl-n-glutamate; 5CH$_2$H$_4$PteGlun, 5-methyltetrahydropteroyl-n-glutamate; 5CHOH$_2$H$_4$PteGlun, 5-formyltetrahydropteroyl-n-glutamate; 10CHOH$_2$H$_4$PteGlun, 10-formyltetrahydropteroyl-n-glutamate; FPGS, folylpolyglutamate synthetase; H$_2$PteGlun, tetrahydropteroyl-n-glutamate; H$_2$PteGlun, dihydropteroyl-n-glutamate; PteGlun, folinic acid (pteroylglutamic acid); D fraction, fraction of the folate pool that is H$_2$PteGlun (folic acid does not form ternary complexes in our system); F fraction, fraction of the folate pool that is 5CHOH$_2$H$_4$PteGlun + 5,10CH$_2$H$_4$PteGlun + 10CHOH$_2$H$_4$PteGlun; M fraction, fraction of the folate pool that is 5CH$_2$H$_4$PteGlun; U fraction, fraction of the folate pool that is H$_2$PteGlun + 5,10CH$_2$H$_4$PteGlun.
(5CHOH$_2$PteGlu) or 10-formyl-[10CHOH$_2$PteGlu] tetrahydrofolate to the methylene oxidation state of 5,10-methylenetetrahydrofolate (5,10CH$_2$H$_2$PteGlu) to the fully reduced methyl state of 5-methyltetrahydrofolate (5CH$_3$H$_2$PteGlu). Each of these pteridine derivatives can also have up to eight or nine glutamates usually found in gamma peptide linkage as a part of the biological folate (some bacteria have alpha-linked glutamates past the third glutamate). This variability in the forms of folate in biological systems presents a challenge in monitoring the folate pools in tissues, especially in tissues in which the concentrations of folates are relatively low.

In liver, where the concentrations of folate are higher than in other tissues, the folates are localized in the cytoplasmic and the mitochondrial fractions [Carl et al. 1995, Cook and Blair 1979, Horne et al. 1989]. The cytoplasmic fraction contains primarily 5CH$_3$H$_2$PteGlu, plus a pool containing 5,10CH$_2$H$_2$PteGlu, + H$_2$PteGlu, and the mitochondrial pool contains primarily formyl-substituted folates [Carl et al. 1995, Cook and Blair 1979, Horne et al. 1989]. The polyglutamate chain length of the cytoplasmic pool of liver seems to be slightly longer than the chain length of the mitochondrial pool [Carl et al. 1995], with the cytoplasmic pool containing primarily hexaglutamates and the mitochondrial pool primarily pentaglutamates.

Brain contains much lower concentrations of folate than does liver (Cossins 1984), but brain is resistant to folate deficiency [Carl and Smith 1983 and 1984]. The polyglutamate tail of the folates has been associated with the retention of folate within the cell [Shane 1989], but triglutamates have been shown to be adequate for retention and metabolism as long as the triglutamates are synthesized in the mitochondria [Lin and Shane 1994]. Mammalian cells contain folylpolyglutamates that are primarily in the penta- to heptaglutamate range, with the longer chains associated with lower folate concentrations [Shane 1989]. The addition of glutamates to folates is slow [Cook et al. 1987], and folylpolyglutamate synthetase (FPGS) prefers specific pteridine forms [Cichowicz and Shane 1987] that are unequally distributed between cytoplasmic and mitochondrial pools in liver [Carl et al. 1995, Cook and Blair 1979, Horne et al. 1989]. Folylpolyglutamate synthetase activity is found in both cytoplasm and mitochondria [Lin et al. 1993]. With all of these factors contributing to the composition of the subcellular pools of folylpolyglutamates, it is difficult to predict the subcellular distribution of folylpolyglutamates in brain.

In addition, because the regulation of one-carbon metabolism in the brain is different from that in the liver [e.g., the activities of betaine:homocysteine methyltransferase (McKeever et al. 1991) and glycine-N-methyltransferase (Yeo and Wagner 1994), both cytoplasmic enzymes, and dimethylglycine dehydrogenase and sarcosine dehydrogenase (Wagner and Wittwer 1981), both mitochondrial enzymes, are apparently absent from the brain], it might also be expected that the distribution of the derivatives of the pteridine moiety would be different in the brain compared with the liver. To answer these questions we examined the subcellular distribution of folate derivatives in rat brain. We report here the results of that study.

**METHODS**

**Materials.** Chemicals and enzymes were obtained as previously described [Carl and Smith 1995, Carl et al. 1995].

**Animals.** Males were obtained from our local colony of Wistar-derived rats. These animals have been bred and maintained locally for 30 y. The rats were allowed free access to food (Teklad Rodent Diet 8604, Harlan Teklad, Madison, WI, with 1.6 mg folic acid/kg diet) and water. The animal room was maintained at 22°C and 40–60% humidity, with a 12-h light-dark cycle. Five 4-mo-old rats were killed by asphyxiation with CO$_2$ according to procedures approved by the Animal Use Committee of the VA Medical Center, Augusta, GA.

**Tissue preparation.** Brains were excised immediately, weighed, homogenized and fractionated by differential centrifugation using a modification of the method used for liver fractionation as described previously [Carl et al. 1995]. For brain fractionation the method was modified by excluding the isolation of nuclei from the procedure and by adding a 5% Ficoll layer to the gradient for separation of the components of the crude mitochondrial fraction (12,000 × g pellet). Because nuclei from liver did not contain measurable concentrations of folate [Carl et al. 1995], and because the nuclei from brain are difficult to isolate, and because brain is essentially a nonproliferating tissue, making it unlikely that brain nuclei would contain significant folate-dependent activities, we decided that attempting to determine folate in brain nuclei would not be worthwhile. A 5% Ficoll layer was added to the gradient used to separate the particulate components of the crude mitochondrial fraction in order to facilitate the separation of these components, which are present in much higher concentrations in brain than in liver. Only the pellet and the layers floating on the 7.5% and the 10% Ficoll were collected and assayed.

**Folate analysis.** The compositions of the folate pools in the various fractions of brain were determined by a modification of our standardization of the method of Priest and co-workers [see Carl and Smith 1995 for review]. To assay subcellular fractions of brain for folate derivatives, the volumes of the samples were increased from a maximum of 50 µL to a maximum of 200 µL, and the total volume of the incubation for conversion of the folates to 5,10CH$_2$H$_2$PteGlu, was increased from 100 to 250 µL. Twenty microliters of each incubated sample was treated in triplicate with 5 µL
RESULTS

Our analysis of rat brain folate pools indicated that the pteridine derivatives of the brain folates were distributed in a manner very similar to their distribution in liver (Carl et al. 1995). The primary folates in brain were the U fraction \( \text{H}_4\text{PteGlu}_n \), the F fraction \( 5\text{CH}_4\text{H}_4\text{PteGlu}_n \) and the M fraction \( 5\text{CH}_5\text{H}_4\text{PteGlu}_n \) [Fig. 1]. The F fraction \( 5\text{CHOH}5\text{CH}_4\text{H}_4\text{PteGlu}_n \) is also present in substantial concentrations, and even oxidized forms of folate \( \text{D} \) fraction \( \text{H}_4\text{PteGlu}_n \) contributed substantially to the total brain folate pool. It is interesting that the formylfolates in the whole-brain homogenate were characterized by a slightly longer polyglutamate chain than was the \( 5\text{CH}_3\text{H}_4\text{PteGlu}_n \). This observation is different from that in the liver (Carl et al. 1995), in which the \( 5\text{CH}_3\text{H}_4\text{PteGlu}_n \) found in the cytoplasm had a longer polyglutamate tail than did the formylfolates. Brain cytoplasm \( 100,000 \times g \) supernatant, like liver cytoplasm (Carl et al. 1995), contained much higher concentrations of the \( 5\text{CH}_3\text{H}_4\text{PteGlu}_n \) than of any of the other folates [Fig. 2]. Indeed, the formylfolates and the oxidized folates were present at combined levels of only about 20% of the \( 5\text{CH}_3\text{H}_4\text{PteGlu}_n \) concentration. The polyglutamate distribution in the cytoplasmic fraction showed an almost equal distribution between the penta- and hexaglutamates, a distribution similar to

![FIGURE 1](https://academic.oup.com/jn/article-abstract/126/12/3077/4724676)

![FIGURE 2](https://academic.oup.com/jn/article-abstract/126/12/3077/4724676)

- **FIGURE 1** The composition of the folate pool in homogenates of rat brain. Rats were killed by \( \text{CO}_2 \) asphyxiation, and the brain was immediately excised and homogenized in nine volumes of buffered isotonic sucrose. Each bar represents the mean \( \pm \) SEM of folate analyses of the brains of five rats. Folates were determined by the ternary complex method and protein by the method of Lowry. The pool designated U consists of \( \text{H}_4\text{PteGlu}_n + 5\text{CH}_4\text{H}_4\text{PteGlu}_n \), the F pool of \( 5\text{CHOH}5\text{CH}_4\text{H}_4\text{PteGlu}_n \) and \( 5\text{CH}_4\text{H}_4\text{PteGlu}_n + 10\text{CH}_2\text{H}_4\text{PteGlu}_n \), and the M pool of \( 5\text{CH}_3\text{H}_4\text{PteGlu}_n \).

- **FIGURE 2** The composition of the folate pool in the cytoplasmic fraction of rat brain. Rats were killed by \( \text{CO}_2 \) asphyxiation, and the brain was immediately excised and homogenized in nine volumes of buffered isotonic sucrose. Cytoplasm was generated by differential centrifugation. The supernatant from a 1-h spin at 100,000 \( \times g \) was designated cytoplasm. Each bar represents the mean \( \pm \) SEM of folate analyses of the cytoplasmic fractions of the brains of five rats. Folates were determined by the ternary complex method and protein by the method of Lowry. The pool designated U consists of \( \text{H}_4\text{PteGlu}_n + 5\text{CH}_4\text{H}_4\text{PteGlu}_n \), the D pool of \( 5\text{CHOH}5\text{CH}_4\text{H}_4\text{PteGlu}_n \) and \( 5\text{CH}_4\text{H}_4\text{PteGlu}_n + 10\text{CH}_2\text{H}_4\text{PteGlu}_n \), and the M pool of \( 5\text{CH}_3\text{H}_4\text{PteGlu}_n \).
The cot, folate dehydrogenase/cytochrome of heptaglutamates. plasmic portion the hexaglutamates of chondria the of ties though that glutamate chain

that in liver (Carl et al. 1995). However, in brain mitochondria (gradient pellet), which exhibited higher concentrations of the formyl and unsubstituted folates (as did liver mitochondria; Carl et al. 1995), the polyglutamate chain lengths were longer than those in the cytoplasm (Table 1) unlike liver; Carl et al. 1995), exhibiting almost equal concentrations of hexa- and heptaglutamates in the formyl derivatives and primarily hexaglutamates in the unsubstituted fraction (Fig. 3).

The distribution of the pteridine and polyglutamate derivatives in the crude synaptosomal fraction [layer floating on 7.5% Ficoll] (Fig. 4) initially seemed to support the hypothesis that the crude synaptosomal fraction was simply a mixture of mitochondrial and cytoplasmic folates. Indeed, the distribution pattern of the crude synaptosomal folates was very similar to that of the homogenate and might be considered an additive composite of the mitochondrial and cytoplasmic distribution patterns. However, the oxidized pteridine fraction [D] showed appreciable levels in the homogenate fraction and the crude synaptosomal fraction, exhibiting measurable concentrations of penta-, hexa- and heptaglutamates. The oxidized folate fraction was substantially smaller in the mitochondrial and cytoplasmic fractions and showed very different distributions of the polyglutamate derivatives. The activities of the marker enzymes for mitochondria [cytochrome oxidase] and cytoplasm [lactate dehydrogenase] indicated that the gradient pellet was enriched in mitochondria and that the 12,000 × g supernatant and the 100,000 × g supernatant exhibited high ratios of lactate dehydrogenase/cytochrome oxidase activities (Table 2). The 12,000 × g supernatant showed a nearly identical folate composition to that in the 100,000 × g supernatant, which is presented here as cytoplasm.

Folate concentrations were greater, as expected, in the mitochondrial (gradient pellet) and cytoplasmic (100,000 × g supernatant) fractions than they were in the homogenate (Table 2). The crude synaptosomal fraction (7.5% Ficoll layer) exhibited total folate concentrations intermediate between those of the enriched mitochondrial fraction and the enriched cytoplasmic fraction (Table 2), as might be expected if the synaptosomal fraction were a mixture of these two fractions. However, the difference between the synaptosomal folate D pool and the folate D pools of the cytoplasmic and mitochondrial fractions indicates that synaptosomal folate is not just a mixture of cytoplasmic and mitochondrial folates.

**TABLE 1**

<table>
<thead>
<tr>
<th>Glutamate chain length</th>
<th>Fraction of compartmental folate represented by designated polyglutamate</th>
<th>Cytoplasm</th>
<th>Mitochondria</th>
<th>P &lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.342 ± 0.022</td>
<td>0.199 ± 0.031</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.345 ± 0.037</td>
<td>0.318 ± 0.060</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.124 ± 0.022</td>
<td>0.253 ± 0.027</td>
<td>0.0005</td>
<td></td>
</tr>
</tbody>
</table>

1 The fraction of each folate pool [cytoplasmic and mitochondrial] that is represented by each specific polyglutamate was calculated. These fractions were averaged and the variances calculated. Although cytoplasmic and mitochondrial folate pools are separate entities and cannot be directly compared, we compared the proportions of each pool represented by specific polyglutamate chain lengths to determine whether the differences observed were greater than the inherent error of the method. The P values in the last column indicate that the differences seen are greater than the inherent error of the method. Values are means ± SEM. n = 5.

**DISCUSSION**

The subcellular folate distribution described here supports the hypothesis of Barlowe and Appling (1988) that one-carbon units are generated in the mitochondria by the folate-dependent formation of formate, which is then transported to the cytoplasm to be used in generating formyl, methylene or methyl groups for synthetic purposes. The localization of the formylfolates, primarily in the mitochondria, and methylfolate,

**FIGURE 3** The composition of the folate pool in the mitochondrial fraction of rat brain. Rats were killed by CO2 asphyxiation, and the brain was immediately excised and homogenized in nine volumes of buffered isotonic sucrose. Mitochondria were isolated by differential centrifugation. The 12,000 × g pellet was resuspended and layered on a Ficoll gradient (5%-7.5%-10% in buffered isotonic sucrose) and centrifuged for 1 h at 100,000 × g. The pellet that formed below the gradient was designated mitochondria. Each bar represents the mean ± SEM of folate analyses of the mitochondrial fractions of the brains of five rats. Folates were determined by the ternary complex method and protein by the method of Lowry. The pool designated U consists of H₂PteGlu₉ + 5,10CH₂H₂PteGlu₉, the pool D of H₂PteGlu₉, the pool F of 5CHOH₂PteGlu₉ + 5,10CH⁺H₂PteGlu₉ + 10CHOH₂PteGlu₉, and the M pool of 5CH₃H₂PteGlu₉.
primarily in the cytoplasm, is consistent with this hypothesis. It is intriguing that the subcellular distribution of folates in the brain is so similar to the subcellular distribution of folates in the liver, because the liver contains several enzymes of one-carbon metabolism, including several folate-binding proteins, that are not present in brain. For example, glycine-N-methyltransferase, a cytoplasmic enzyme that strongly binds 5CH3H2PteGlu (Wagner et al. 1985), is present in large concentrations in the liver but is absent from the brain. Betaine:homocysteine methyltransferase, an enzyme of choline catabolism that salvages a methyl group in a reaction that forms methionine, is present in liver cytoplasm but not in brain cytoplasm (McKeever et al. 1991). Dimethylglycine dehydrogenase and sarcosine dehydrogenase are two more enzymes in the choline catabolic pathway that oxidize a methyl group from their respective substrates to form 5,10CH2H3PteGlu. These enzymes are present in liver mitochondria but not brain mitochondria (Wagner and Wittwer 1981). Considering the suggestion of Schirch and Strong (1989) that cellular folates exist primarily bound to proteins, it is surprising that—with the differences in the folate-dependent protein complement between the tissues—the subcellular distribution of the pteridine derivatives of the folates is so similar. Indeed, except for the concentrations (brain folate concentration is about 5% of the folate concentration in the liver), the relative distribution of the pteridine derivatives is nearly identical. Subcellular compartmentation of folate metabolism has recently been reviewed (Wagner 1996).

The polyglutamate derivatives, on the other hand, were longer in the brain mitochondria than in the liver mitochondria (Carl et al. 1995), but very similar in the brain cytoplasm and liver cytoplasm (Carl et al. 1995). Mechanisms for the regulation of the lengths of the polyglutamate chains are unknown. It has been suggested that folate enters cells primarily in the form 5CH3H2PteGlu (Horne 1990, Horne et al. 1979, 1992b and 1993) and that folate enters the mitochondria pri-

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**Table 2**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Folate pmol/mg protein</th>
<th>Cytochrome oxidase μmol (min · mg protein)−1</th>
<th>Lactate dehydrogenase μmol (min · mg protein)−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate*</td>
<td>3.52</td>
<td>4.95 ± 0.58</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>3000 × g pellet</td>
<td>3.52 ± 0.51</td>
<td></td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>3000 × g supernatant</td>
<td>3.73 ± 0.53</td>
<td></td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>12,000 × g pellet</td>
<td>5.88 ± 0.47</td>
<td></td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>7.5% Ficoll layer*</td>
<td>5.16</td>
<td>1.81 ± 0.29</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>10% Ficoll layer</td>
<td>5.15 ± 0.74</td>
<td></td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>Gradient pellet*</td>
<td>4.39</td>
<td>16.32 ± 1.98</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>12,000 × g supernatant</td>
<td>0.73 ± 0.31</td>
<td></td>
<td>1.13 ± 0.01</td>
</tr>
<tr>
<td>100,000 × g pellet</td>
<td>1.11 ± 0.19</td>
<td></td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>100,000 × g supernatant*</td>
<td>5.96</td>
<td>0.23 ± 0.12</td>
<td>0.53 ± 0.04</td>
</tr>
</tbody>
</table>

1 The numbers represent means ± SEM of activities measured in fractions from five separate fractionations. Fractions were generated by differential centrifugation as described in Methods.
2 Total folate concentrations were calculated by summing the individual folate concentrations. Asterisks indicate fractions for which folate distribution is shown in Figures 1-4.
3 Cytochrome oxidase activity was used as a marker for mitochondria, and lactate dehydrogenase activity was used as a marker for cytoplasm.
Carly in the form 5CHOH₂PteGlu₃ (Horne et al. 1992a). It has been shown that folylpolyglutamyl synthetase (FPGS) is present in both cytoplasm and mitochondria (Lin et al. 1993). Therefore, it might be expected that the polyglutamates in each fraction are synthesized there and that the longer chains might indicate a longer half life as the FPGS slowly adds additional glutamates to the molecule. The longer polyglutamate chains in the mitochondria may also result from the fact that the mitochondrial folates are primarily H₂PteGlu₃ and CHOH₂PteGlu₃, which are better substrates for FPGS than is the primary cytoplasmic folate 5CH₃H₂PteGlu₃ (Shane 1989).

The presence of the oxidized folates with similar polyglutamate patterns in the homogenate and crude synaptosomal fractions and the absence of these patterns from the mitochondrial and cytoplasmic fractions indicate that there may be a function for oxidized folates in the brain that is not linked to either mitochondria or cytoplasm. The appearance of these folates in the crude synaptosomal fraction suggests a possible role in synaptic function, but because these preparations were relatively crude, the evidence is at most suggestive.

ACKNOWLEDGMENTS

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LITERATURE CITED


