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The metabolism of 5-methyltetrahydropteroyl-L-glutamic acid and its oxidation products in the rat

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Folate metabolism in the rat was investigated using radiolabelled 5-methyltetrahydropteroylglutamate (5-CH_3-H_4PteGlu) and its oxidation products. 5-CH_3-H_4PteGlu is absorbed completely from the intestine, although in some preparations it is an equimolecular mixture of C-6 epimers, only one of which is naturally present in biological systems. The methyl group is incorporated into non-folate compounds, including methionine and creatine. No evidence was observed for the oxidation of the methyl group of 5-CH_3-H_4PteGlu to form other folate types. The tetrahydrofolate moiety of 5-CH_3-H_4PteGlu is metabolized in a similar manner to folic acid, forming formyl folates and tissue polyglutamates, and is catabolized by scission. The triazine oxidation product of 5-CH_3-H_4PteGlu is not metabolized by the rat or its gut microflora. 5-Methyl-5,6-dihydropteroylglutamate, however, is assimilated into the folate pool, but is substantially broken down by passage through the gut. The possible implication of this in scorbutic diets is discussed.

Most folate metabolic studies have used PteGlu tracers because of their commercial availability and relative stability. However, as this compound is not a naturally occurring folate form, it is possible that its use may be inappropriate. We report experiments on the rat with radiolabelled 5-CH_3-H_4PteGlu, the major folate monoglutamate of human serum (Ratanasthien *et al.*, 1977) and its two characterized oxidation products 5-CH_3-H_2PteGlu and a compound previously described as 4a-hydroxy-5-methyltetrahydropteroyl-L-glutamic acid. The studies of Jongejan *et al.* (1979) on the H_2O_2 oxidation of tetrahydropteroyl-L-glutamate analogues suggest the latter compound is in fact a pyrazino-*s*-triazine derivative. These two compounds are likely constituents of foodstuffs, owing to oxidation of the folate content (Blair, 1976). 5-$^{14}CH_3$-H_4PteGlu was used to observe the role of 5-CH_3-H_4PteGlu as a one-carbon donor, and mixed-label (2-^{14}C)- plus (3′,5′,7,9-^{3}H)-labelled 5-CH_3-H_4PteGlu was used to follow the disposition of the tetrahydrofolate moiety.

Abbreviations used: PteGlu, pteroyl-L-glutamic acid; 5-CH_3-H_4PteGlu, 5-methyltetrahydropteroyl-L-glutamic acid; 5-CH_2-H_2PteGlu, 5-methyl-5,6-dihydropteroyl-L-glutamic acid.

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Materials and methods

Chemicals

5-$^{14}CH_3$-H_4PteGlu (barium salt; sp. radioactivity 58 Ci/mol), [2-^{14}C]PteGlu (sp. radioactivity 55 Ci/mol) and [3′,5′,7,9-^{3}H]PteGlu (sp. radioactivity 45 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and 5-CH_3-H_4PteGlu (magnesium salt) was from Eprova Research Laboratories (Basle, Switzerland). Mixed-label naturally occurring diastereoisomeric [2-^{14}C,3′,5′,7,9-^{3}H]5-CH_3-H_4PteGlu was isolated by DEAE-cellulose ion-exchange chromatography from the urine of rats orally dosed with similarly labelled PteGlu. The 5-CH_3-H_4PteGlu fraction was desalted on a Sephadex G-15 column (Pharmacia) equilibrated with 0.2% sodium ascorbate. The two oxidation products of 5-CH_3-H_4PteGlu were prepared by modifications of the methods of Gapski *et al.* (1971), and purified by chromatography on DEAE-cellulose and Sephadex G-15. 5-CH_3-H_2PteGlu was prepared from mixed-labelled 5-CH_3-H_4PteGlu and the triazine from 5-$^{14}CH_3$-H_4PteGlu. All prepared radioactive compounds chromatographed as single peaks on DEAE-cellulose ion-exchange (DE-52) chromatography and had u.v. absorption spectra in agreement with those described in the literature (Gapski *et al.*, 1971).

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Animals

Male Wistar rats (200g) in groups of four to six were dosed either orally or intraperitoneally with up to 2μCi of ^{14}C or 5μCi of ^{3}H in 50mM-sodium phosphate buffer, pH 7.0, containing 2% sodium ascorbate, or N_2-gassed water for 5-CH_3-H_2PteGlu solutions. The animals were placed in glass metabolism cages ('Metabowl'; Jencons, Hemel Hempstead, Herts., U.K.), which facilitated separate collection of expired CO_2 (in 100cm^3 of 2M-NaOH), urine and faeces. Throughout the experiment the animals had access to food and water *ad libitum*. Urine was collected into foil covered flasks containing 100mg of sodium ascorbate and 5cm^3 of phosphate buffer, pH 7.0, for periods of 0–6h, 6–24h, 24–48h and 48–72h after administration. Faeces and CO_2 were collected daily. After 3 days the rats were killed, and the liver, kidneys, spleen, gut and some leg muscle were excised. One liver from each group was extracted in boiling 2% (w/v) sodium ascorbate in 50mM-phosphate buffer, pH 7.0, to determine the nature of the retained folates (Barford *et al.*, 1977). Samples of muscle were extracted in 50mM-phosphate buffer, pH 7.0.

Determination of radioactivity

Radioactivity in urine samples and column effluents was determined as described by Connor *et al.* (1979) and that in freeze-dried tissue samples and faeces as described by Barford *et al.* (1978).

Chromatography

Urine samples were sequentially chromatographed on DEAE-cellulose ion exchange resin (DE-52; Whatman, Maidstone, Kent, U.K.) and gel-filtration columns (Sephadex G-15; Pharmacia) as described previously (Barford *et al.*, 1977). Hot ascorbate extracts of livers were chromatographed first on Sephadex G-15; the collected peaks were then chromatographed on DEAE-cellulose columns. Radioactive compounds were identified by co-chromatography with authentic standards on both DEAE-cellulose and Sephadex chromatography.

In addition, paper chromatography was used to resolve ^{3}H-labelled folate scission products (Connor *et al.*, 1979).

Because of the low concentrations of the radioactive species in rat tissues and urine (1μM–10nM) and their intractable chemical nature (no melting point, no uniquely characteristic mass spectra, i.r. spectra or u.v. spectra) no criteria other than chromatographic behaviour could be used for identification.

Results

5-$^{14}CH_3$-H_4PteGlu

Four male rats were dosed orally with 5-$^{14}CH_3$-H_4PteGlu (80μg/kg body wt.; 2μCi). Table 1 gives the distribution of retained and excreted ^{14}C radioactivity. The excretion of ^{14}C in faeces was low, indicating that the compound was absorbed in excess of 97% from the gut, although synthetic 5-CH_3-H_4PteGlu is an equimolar mixture of the naturally occurring and unnatural C-6 epimers. No significant difference in faecal excretion was seen after intraperitoneal administration.

DEAE-cellulose chromatography of the urine separated the ^{14}C radioactivity into two fractions (Table 1). The major peak co-chromatographed with 5-CH_3-H_4PteGlu in both column systems: the minor peak eluted at below 0.1M-NaCl on DEAE-cellulose, a position inconsistent with known folates and was termed the non-folate fraction. This peak rechromatographed on Sephadex G-15 with both methionine and creatine. After acidification to pH 1 (which dehydrates creatine to creatinine), 38% of the non-folate-fraction activity rechromatographed with creatinine, with 56% remaining at the methionine position.

No ^{14}C was found associated with folates other than 5-CH_3-H_4PteGlu when 5-$^{14}CH_3$-H_4PteGlu and [3′,5′,7,9-^{3}H]PteGlu were administered simultaneously (Kennelly, 1980).

Of the ^{14}C applied, 50.9% was excreted in the first-day urine as 5-CH_3-H_4PteGlu, which suggests there may be specific excretion of the unnatural C-6 epimer. However, 5-CH_3-H_4PteGlu isolated from the 0–24h urine sample when re-administered to rats, showed similar formation of non-folate fraction and retention in tissue (Table 2) to that of stock 5-$^{14}CH_3$-H_4PteGlu, indicating that they have similar bio-availability, whereas that extracted from the 24–48h urine sample was excreted unchanged.

Retention of ^{14}C radioactivity after 3 days was low in liver and kidney. Chromatography of the liver extract showed no evidence of radiolabelled folate polyglutamates, the retained radioactivity eluting from DE-52 DEAE-cellulose at less than 0.1M-NaCl, whereas pteroylpoly-L-glutamates elute at values greater than 0.5M-NaCl. The greatest tissue retention was in muscle (Table 1). Chromatography of the muscle extract showed 21% of ^{14}C eluting as high-molecular-weight compounds, presumably protein, whereas 69% eluted at the position of urinary non-folate fraction. After acidification, 93% of this activity co-eluted with creatinine.

Mixed-label natural diastereoisomeric [2-^{14}C,3′,5′,7,9-^{3}H]5-CH_3-H_4PteGlu

Two groups of rats were dosed orally with [2-^{14}C,3′,5′,7,9-^{3}H]5-CH_3-H_4PteGlu (8 and 80μg/kg body wt. respectively). Table 3 gives the distribution of retained and excreted radioactivity. At 80μg/kg body wt. the total faecal radioactivity is markedly increased compared with the *Me*-^{14}C-labelled species, a 3-day total of 30.2% ^{14}C and

Table 1. *The distribution of radioactivity recovered from four rats after the oral administration of 5-$^{14}CH_3$-H_4PteGlu (80 μg/kg body wt.)*

The results are expressed as percentage of dose (means ± S.E.M.; $n = 4$ for urine and faeces; mean of two samples for others).

	Distribution of radioactivity (% of dose)		
	0–24 h	24–48 h	48–72 h
Urine	50.9 ± 4.0	1.9 ± 0.2	1.2 ± 0.2
As non-folate fraction	3.0	0.55	0.42
As 5-CH_3-H_4PteGlu	47.9	1.07	0.42
Faeces	2.3 ± 1.0	0.1 ± 0.03	0.02 ± 0.01
CO_2	2.0	0.4	0.4
Liver			0.1
Muscle*			13.2
Kidney			0.1
Spleen			0.01
Total	55.2	2.4	15.0

* Calculated, assuming muscle = 40% body weight.

Table 2. *Distribution and excretion of stock 5-$^{14}CH_3$-H_4PteGlu and re-administered urinary 5-$^{14}CH_3$-H_4PteGlu radioactivity after oral administration to the rat*

For the results for the recovery of the administered dose, those for urine are means ± S.E.M. and those for spleen, muscle, kidney and liver are means of two samples.

	Urine extract	Stock compound
Number of animals	3	7
Dose (μg/kg body wt.)	1.25	2.0
Proportion of dose recovered (%) in:		
Urine 0–24 h	12.2 ± 0.4	10.8 ± 1.4
24–48 h	3.8 ± 0.4	4.2 ± 0.5
Spleen	1.6	0.1
Muscle*	4.6	6.8
Kidney	2.2	1.6
Liver	3.0	4.5
Proportion of radioactivity (%) in 0–24 h urine as:		
Non-folate fraction	17.6	22.1
5-CH_3-H_4PteGlu	71.3	68.3

* Calculated assuming muscle = 40% body weight.

Table 3. *The distribution of radioactivity recovered after oral administration of [2-^{14}C,3',5',7,9-^{3}H]5-CH_3-H_4PteGlu*

The results are expressed as percentages of the dose (means ± S.E.M. for urine and faeces; means of two samples for others). Abbreviation: ND, not determined.

	Distribution of radioactivity (% of dose)			
Dose (μg/kg body wt.) ...	80 ($n = 4$)		8 ($n = 5$)	
	^{14}C	^{3}H	^{14}C	^{3}H
Urine				
0–6 h	19.2 ± 3.3	17.5 ± 3.0	39.1 ± 2.9	41.0 ± 5.9
6–24 h	23.2 ± 3.6	24.5 ± 3.4	28.5 ± 4.2	40.4 ± 5.5
24–48 h	2.8 ± 0.6	4.0 ± 0.5	3.2 ± 0.3	4.7 ± 0.4
48–72 h	2.6 ± 0.2	2.7 ± 0.1	3.4 ± 0.6	6.2 ± 1.2
Faeces	30.2 ± 3.0	21.5 ± 1.9	27.1 ± 2.0	22.0 ± 1.8
Liver	2.5	2.4	6.8	ND
Kidney	0.1	0.3	0.8	0.4
Gut	0.0	0.0	1.1	0.8
Total	80.6	70.2	110.0	115.5

Table 4. *Metabolites detected in the 6–24 h urine of rats given an oral dose of* [2-^{14}C,3',5',7,9-^{3}H]5-CH_3-H_4PteGlu

Dose (μg/kg body wt.) ...	Proportion of radioactivity (% of dose)			
	80		8	
	^{14}C	^{3}H	^{14}C	^{3}H
10-Formyl-PteGlu	4.4	4.4	3.1	3.7
5-CH_3-H_4PteGlu	6.7	6.9	8.4	12.5
Unknown folate	4.8	4.2	5.3	7.7
p-Acetamidobenzoate		3.0		5.5
p-Acetamidobenzoyl-L-glutamate		2.7		10.7

21.5% ^{3}H compared with 2.4% of 5-$^{14}CH_3$-H_4PteGlu. As the very low faecal recovery suggests that the methyl-labelled compound is completely absorbed, this would indicate a greater biliary excretion of radioactivity derived from the mixed species. The recovery of radioactivity from both doses of the mixed-label compound shows an excess of ^{14}C over ^{3}H in the faeces and the reverse in urine (Table 3).

Chromatography of the urine showed the presence of the following radioactive species: 10-formyl-PteGlu, 5-CH_3-H_4PteGlu, an unidentified dual-labelled species, *p*-acetamidobenzoyl-L-glutamate and *p*-acetamidobenzoate (for further details of the identifications, see Pheasant *et al.*, 1981). The distribution of these metabolites is given in Table 4. The intact folates excreted in urine showed a higher ^{3}H/^{14}C ratio than the administered compound, thus accounting for 5% of the ^{3}H excess in the 0–24 h urine sample. At both doses, a substantial proportion of the retained activity was observed in liver, gut and kidney (Table 3), proportionally greater retention occurring at 8 μg/kg body wt. Chromatography of the liver extract showed the presence of a radiolabelled high-molecular-weight peak eluting close to the void volume on Sephadex G-15 and at 0.7 M-NaCl on DEAE-cellulose, positions compatible with pteroylpoly-L-glutamate species.

5-CH_3-H_2PteGlu

[2-^{14}C,3',5',7,9-^{3}H]5-CH_3-H_2PteGlu and [3',5',7,9-^{3}H]5-$^{14}CH_3$-H_2PteGlu were used. The former was dosed orally at 0.06 μg/kg body wt. to six rats, and the latter at 0.4 μg/kg body wt. by intraperitoneal injection to another group of six rats. Chromatography of the 0–6 and 6–24 h urine samples showed differing urinary products; after the oral dose only ^{3}H-labelled scission metabolites (48% of the dose) were resolved on DEAE-cellulose, whereas the urine collected from intraperitoneally dosed rats contained intact folates as the majority of the ^{3}H- and ^{14}C-labelled metabolites and [^{14}C]methionine and [^{14}C]creatine. The ^{3}H peaks from the urine after the oral dose were identified as *p*-acetamidobenzoyl-L-glutamate in the 0–6 h sample and *p*-acetamidobenzoate and *p*-acetamidobenzoyl-L-glutamate in the 6–24 h urine sample.

Table 5. *The distribution of radioactivity recovered from rats after oral administration of* [^{14}C]*methyltriazine (41.5 μg/kg body wt.)*

Results are means ± S.E.M. for urine and faeces and means of two samples for other tissues.

	Proportion of radioactivity recovered (% of dose)
Urine	
0–24 h	35.0 ± 9.6
24–48 h	3.2 ± 0.2
48–72 h	3.5 ± 0.4
Faeces	
0–24 h	24.7 ± 7.4
24–48 h	17.5 ± 1.2
48–72 h	9.0 ± 4.3
Liver	0.5
Kidney	0.5
Spleen	0.1
Muscle*	1.0
Total	95.0

* Calculated as 40% body wt.

[^{14}C]*Methyltriazine*

[^{14}C]Methyltriazine was diluted with unlabelled triazine and dosed orally at 41.5 μg/kg body wt. to four rats. The distribution of ^{14}C radioactivity recovered is given in Table 5. Very little radioactivity was retained in the tissues examined; at this dose 96% ^{14}C was lost via urine and faeces over 3 days. Chromatography of the urines on days 1, 2 and 3 showed a single ^{14}C peak eluting with added triazine on both DEAE-cellulose and Sephadex G-15 columns.

To investigate whether the faecal activity contained triazine metabolites formed by the rat or its gut microflora, a sample of first-day faeces was extracted with 1% NH_3 solution containing 1% (v/v) mercaptoethanol. This procedure extracted 88% of faecal ^{14}C. The neutralized extract gave a single peak on both DEAE-cellulose and Sephadex G-15 columns, inseparable from added triazine.

Discussion

The methyl group of 5-CH_3-H_4PteGlu is observed to be removed by transfer reactions to non-folate compounds that are qualitatively similar to those derived from the methyl group of methionine (Kennelly, 1980). This would suggest that the methyl metabolism of 5-CH_3-H_4PteGlu normally proceeds exclusively via methionine formation, as predicted in the 'methyltetrahydrofolate trap' model of pernicious anaemia first proposed by Herbert & Zalusky (1962) and Norohana & Silverman (1962).

5-$^{14}CH_3$-H_4PteGlu is absorbed completely from the gut, as shown by the low faecal recoveries, although it is an equimolar mixture of C-6 epimers; this would imply that if absorption proceeds via a carrier mechanism, the carrier does not specifically bind at C-6. Alternatively, absorption might proceed by a non-carrier process (Blair *et al.*, 1975*a*; Coleman *et al.*, 1979).

The experiments with 5-CH_3-H_4PteGlu labelled in the folate skeleton show that this portion of the molecule is assimilated into the folate pool, as shown by the formation of formylfolate in urine and polyglutamates in the tissues, and is degraded by scission to the same catabolites as pteroyl-L-glutamic acid (Murphy *et al.*, 1976; Connor *et al.*, 1979). This is the first report of the catabolism of a naturally occurring reduced folate and supports the model of folate metabolism proposed by Pheasant *et al.* (1981). The observation of elevated $^3H/^{14}C$ ratios in the excreted folates also suggests that the secondary isotope effect observed during the intestinal transport and metabolism of folic acid (Connor *et al.*, 1980) is not restricted to oxidized folates. The incorporation of the folate skeleton into pteroylpoly-L-glutamates is lower than that obtained with pteroyl-L-glutamic acid at similar doses (5-CH_3-H_4PteGlu at 80μg/kg body wt., 2.5%; PteGlu at 100μg/kg body wt., 10%; Pheasant *et al.*, 1981). Also the methyl group of 5-$^{14}CH_3$-H_4PteGlu was not incorporated into polyglutamates, suggesting that demethylation must occur before polyglutamate synthesis. As demethylation provides H_4PteGlu, the most active substrate for polyglutamate synthesis (McGuire *et al.*, 1979), demethylation must be slow compared with polyglutamate synthesis *in vivo*. This is also supported by the urinary excretion of the natural diastereoisomer (Table 2).

5-CH_3-H_4PteGlu is a major food folate and is susceptible to oxidation (Blair *et al.*, 1975*b*). Thus the biological availability of its oxidation products is of nutritional importance. The triazine oxidation product of 5-CH_3-H_4PteGlu was not metabolized by the rat or its gut microflora. It is also inactive for the common microbiological-assay organisms (Gapski *et al.*, 1971) and thus its presence will not give rise to a spurious estimate of the active folate content of foodstuff. 5-CH_3-H_2PteGlu, however, is assimilated similarly to 5-CH_3-H_4PteGlu after intraperitoneal administration but after oral dosage gives rise only to scission products, which indicates it is substantially unavailable if ingested in food. This compound is estimated as 5-CH_3-H_4PteGlu in *Lactobacillus casei* microbiological assays, which include ascorbate (Ratanasthien *et al.*, 1977); thus estimates of food folate content using *L. casei* may be high.

In scurvy occasionally megaloblastic anaemia is observed; Andrews (1977) and Stokes *et al.* (1975) report the abnormal presence of 10-formyl-PteGlu, an oxidation product of 10-formyltetrahydropteroyl-L-glutamate in the urine of a scorbutic subject.

Increased folate oxidation may occur in abscorbate-deficient diets due to deficiency of the natural antioxidant, and 5-CH_3-H_2PteGlu may be formed.

It is possible that this contributes to the folate deficiency in scurvy.

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