The Urinary Excretion of Orally Administered Pteroyl-L-glutamic Acid by the Rat

By J. A. BLAIR AND E. DRANSFIELD

Department of Chemistry, University of Aston, Gosta Green, Birmingham B4 7ET, U.K.

(Received 25 January 1971)

1. The urinary excretion of folates after oral administration of [2-¹⁴C]pteroyl-L-glutamic acid was studied by assaying the radioactivity in the urine and in materials purified and characterized by t.l.c. 2. Radioactivity excreted was 6.8, 5.9 and 30.7% of the oral dose in the first 24 h after doses of 3.1, 32 and 320 µg/kg respectively. 3. Extensive decomposition of urinary folates to pteroyl-L-glutamic acid was prevented by antioxidants or collection of urine frozen. 4. At the three dosages, two major and one minor radioactive compounds were isolated. One of the major metabolites was 5-methyltetrahydropteroylglutamic acid. The others were unidentified but were not pteroylglutamic acid, 7,8-dihydro-, 5,6,7,8-tetrahydro-, 5- or 10-formyl-tetrahydro-, 5,10-methyldiene-tetrahydro-, 5-formimidoyl-tetrahydro-, 5,10-methylene-tetrahydro-, 5-methyltetrahydro-pteroylglutamic acid, nor any decomposition products of these compounds formed during isolation. Labelled unconjugated pteridines were absent. 5. Labelled pteroyl-L-glutamic acid was displaced by oral administration of unlabelled pteroyl-L-glutamic acid (1.6 mg/kg) when given 3.5 h after, but not when given 24 h after the labelled dose. 6. The results show that orally administered [2-¹⁴C]pteroyl-L-glutamic acid is absorbed without metabolism and is then metabolized into naturally occurring tetrahydro-folates. 7. These findings are discussed with reference to previous work.

Since 1935 (Decastello, 1935) a vast amount of literature has been published about the urinary excretion of folates and pteridines, not only for their interest *per se*, but also to provide possible insight into the folate relationships in normal and diseased metabolism.

Of these reports only a few are concerned with the non-microbiological identification of pteridines and folates. These reports include the isolation of uropterin (later identified as xanthopterin) (Koschura & Hrubesch, 1939), isoxanthopterin (Blair, 1958), biopterin (Broquist & Albrecht, 1955; Patterson, Broquist, Albrecht, Saltza & Stokstad, 1955; Patterson, Saltza & Stokstad, 1956), neopterin (Sakurai & Goto, 1967), PteGlu* (Baker *et al.* 1965; Anderson, Belcher, Chanarin & Mollin, 1960) and 5,10-CH=H₄PteGlu (Silverman, Ebaugh & Gardiner, 1956; Albrecht & Broquist, 1956).

By far the largest amount of literature, however,

deals with the microbiological assay of urinary folates. Microbiological assays suffer from several disadvantages, chiefly in their lack of specificity (Blakley, 1969), and have been much criticized (Wieland, Hutchings & Williams, 1952; Johns, Sperti & Burgen, 1961; Kinneary, Johns, MacIntosh, Burgen & Cameron, 1963). This lack of specificity is particularly important when the compound is not characterized by other methods or when impurities are present. It is therefore desirable to develop a simple non-microbiological assay technique for urinary folates. The use of radioactive labelling is advantageous in the study of urinary folates because of the presence of only trace amounts. Tritiated folic acid, first used in 1960 (Anderson *et al.* 1960; Burgen, 1961), although having the main advantages of high specific radioactivity and that it may be used for folate study in man, suffers from the disadvantage of non-specific labelling (Anderson *et al.* 1960; Johns *et al.* 1961; Burgen, 1961; Zakrzewski, Evans & Phillips, 1970) and has been criticized on the grounds of possible impurities (Blakley, 1969).

We now report the results of our studies on the urinary excretion of orally administered

* Abbreviations: PteGlu, pteroyl-L-glutamic acid; H₂PteGlu and H₄PteGlu, 7,8-dihydro- and 5,6,7,8-tetrahydro-pteroyl-L-glutamic acid; 5(and 10)-HCO-, 5(and 10)-formyl-; 5,10-CH=, 5,10-methyldiene-; 5,10-CH₂-, 5,10-methylene-; 5-CH₃-, 5-methyl-; 5-HCNH-, 5-formimidoyl- derivatives.

[2-¹⁴C]PteGlu in the rat, by using the labelling to provide accurate determination of the amounts excreted and t.l.c. to purify and identify the compounds.

MATERIALS

Animals and chemicals. The animals used were adult male Wistar rats, weighing 200–350g, purchased from Scientific Products Farm, Ash, Canterbury, Kent, U.K. Food was diet 41B, supplied by Pilsbury, Edgbaston, Birmingham, U.K. Radiochemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The specific radioactivity of [2-¹⁴C]pteroyl-L-glutamic acid, as the dipotassium salt, was 55.3 mCi/mmol and that of [1-¹⁴C]hexadecane was 1.06 μ Ci/g. Pteridines and pteroyl-L-glutamic acid were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. H₂PteGlu and H₄PteGlu were freshly prepared by the methods of Futterman (1957) and Hatefi, Talbert, Osborn & Huennekens (1960) respectively. Calcium leucovorin was a gift of Lederle Laboratories, Pearl River, N.Y., U.S.A. 5-CH₃-H₄PteGlu was prepared by the method of Blair & Saunders (1970) and 5,10-CH₂-H₄PteGlu by a modification of that method. Characterization and purity of the compounds were periodically determined by t.l.c. and/or by u.v. spectroscopy (Pye Unicam SP.700).

The liquid scintillators used were NE220 (Nuclear Enterprises, Edinburgh, U.K.) and a toluene-based scintillator containing 4g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene (Koch–Light) made up to 1 litre with A.R. toluene.

Antioxidants used were ascorbic acid (British Drug Houses Ltd., Poole, Dorset, U.K.) and 2-mercaptoethanol (Koch–Light). Florisil (60–100 mesh) was purchased

from Koch–Light and cellulose (MN300uv) from Macherey Nagel and Co., Düren, Germany.

Chromatography solvents for t.l.c. These were as follows: (a) butan-1-ol–acetic acid–water (4:1:5, by vol., equilibrated for 20h; upper phase); (b) 0.1M-sodium phosphate buffer, pH7.0; (c) propan-1-ol–aq. 1% (v/v) NH₃ (2:1, v/v); (d) butan-1-ol–acetic acid–water (20:3:7, by vol.); (e) M-formic acid; (f) 0.01M-HCl, pH2.0; (g) 0.2M-sodium acetate buffer, pH4.0; (h) propan-1-ol–butan-1-ol–0.1M-HCl (2:1:1, by vol.); (i) 5% (v/v) acetic acid in water-saturated butan-1-ol; (j) 2% (w/v) ammonium acetate–pyridine (19:1, v/v).

Antioxidant, when used, was 0.5% (v/v) 2-mercaptoethanol for all chromatography solvents and extractions from the cellulose plates (performed with water).

Table 1 shows the characteristic *R_F* values of the pteridines and folates used during this work. Detection levels were of the order of 25 μ g/cm² for light-absorbing compounds and 2 μ g/cm² for fluorescent compounds. The *R_F* values of the compounds when added to urine were distorted and greater attention was paid to the relative *R_F* values, characteristic absorption or fluorescence and co-chromatography.

METHODS

[2-¹⁴C]PteGlu was administered to the rats, the urine was collected, assayed for radioactivity and then purified initially through a Florisil column followed by t.l.c. on cellulose.

Administration of [2-¹⁴C]pteroylglutamic acid. This was administered in 0.5ml of water by stomach intubation to adult male rats. A control rat was given 0.5ml of water only. The animals were housed separately in wire-

Table 1. *Thin-layer chromatography of pteridines and folates*

Samples of fluorescing compounds (1 μ g) and of u.v.-absorbing compounds (10 μ g) were applied in 2 μ l to cellulose plates (250 μ m thick) and developed to 10cm from the origin in solvents containing 0.5% (v/v) 2-mercaptoethanol.

Compound	Characterization under u.v. light (nm)	<i>R_F</i> value		
		Solvent (a)	Solvent (b)	Solvent (c)
2-Amino-4-hydroxypteridine	Blue fluorescence (365)	0.55	0.53	0.43
2-Amino-4-hydroxy-6-formylpteridine	Light-blue fluorescence (365)	0.65	0.68	0.53
2-Amino-4-hydroxypteridine-6-carboxylic acid	Light-blue fluorescence (365)	0.25	0.58	0.12
Xanthopterin	Green fluorescence (365)	0.48	0.61	0.16
Isoxanthopterin	Light-blue fluorescence (365)	0.38	0.40	0.17
Biopterin	Light-blue fluorescence (365)	0.34	0.63	0.49
PteGlu	U.v.-absorbing (254)	0.00	0.50	0.12
10-HCO-PteGlu	Light-blue fluorescence (365)	0.65	0.92	0.30
7,8-H ₂ PteGlu	Light-blue fluorescence (365)	0.55	0.39	0.30
H ₄ PteGlu	U.v.-absorbing (254)	0.43	0.78	0.35
5-HCO-H ₄ PteGlu	U.v.-absorbing (254)	0.69	0.87	0.32
5,10-CH=H ₄ PteGlu	White fluorescence (365)	0.38	0.65	Decomposition
			(tailing)	
5,10-CH ₂ -H ₄ PteGlu	U.v.-absorbing (254)	0.85 (some decomposition)	0.92	0.28
5-CH ₃ -H ₄ PteGlu	U.v.-absorbing (254)	0.68	0.85	0.55

bottomed cages fitted with a urine-faeces separator (Metabowl; Jencons Ltd., Hemel Hempstead, Herts., U.K.). Food and tap water were given *ad libitum*.

Urine collection. This was performed in subdued light every 24 h for up to 7 days after administration of labelled PteGlu. Urines were collected in acid (final pH 1–2), alkali (final concentration 1–2 M-KOH) or frozen (acetone–solid CO₂, –78°C). Antioxidants, when used, were ascorbic acid (final concentration 1–2%, w/v) for urine collected in acid, and 2-mercaptoethanol (final concentration about 0.5%, w/v) for urine collected in alkali. Urines with antioxidant were collected under toluene (5 ml).

Determination of radioactivity. This was done for urines and other aqueous solutions directly by using NE220 liquid scintillator in an automatic scintillation spectrometer (Nuclear Enterprises) operating at 0°C. The background count was approx. 2 c.p.s. Assay of radioactivity in tissues and faeces was performed by combustion (Kalberer & Rutschmann, 1961). The freeze-dried material was ground in a pestle and mortar and a sample (about 50 mg) was burned in 2 litres of oxygen. Carbon dioxide was absorbed in 25 ml of a solution of monoethanolamine (120 ml made up to 1 litre with A.R. methanol) and the radioactivity of a 5 ml sample was counted in 5 ml of toluene-based scintillator. The background count was about 1 c.p.s. The recovery of radioactivity was 96%. [1-¹⁴C]Hexadecane was used as internal standard for each sample. Total sample counts were at least 5000 above background.

Purification by chromatography on Florisil. Florisil (8 g) was washed with 100 ml of aq. 1% (v/v) HCl, then to neutrality with water, followed by 100 ml of aq. 14% (v/v) NH₃, then to neutrality with water and finally with 50 ml of aq. 1% (v/v) HCl. This suspension was packed into a glass column (11.5 cm × 1.3 cm) and shielded from the light. Urine samples were freeze-dried and applied in 25 ml of solution at about pH 1.0. Elution was carried out with 30 ml of aq. 1% (v/v) HCl (acid fraction), followed by 30 ml of water (neutral fraction) and finally 25 ml of aq. 14% (v/v) NH₃ (ammoniacal fraction). Antioxidants, when used, were 2% (w/v) ascorbic acid in acidic solutions and 0.5% (v/v) 2-mercaptoethanol in neutral and alkaline solutions.

There was no significant difference between either the distribution or the recovery of radioactivity from the Florisil column, from urines collected with and without antioxidant, or with and without added folate. The percentage distribution of the radioactivity expressed as the mean ± s.d. for 23 experiments, was 23.9 ± 11.1, 6.8 ± 4.4 and 69.3 ± 13.0 in the acid, neutral and ammoniacal fractions respectively. Recovery of radioactivity was 83.3 ± 14.0%.

Both [2-¹⁴C]PteGlu and 5-CH₃-H₄[¹⁴C]PteGlu when applied in water or urine to a Florisil column were similarly distributed between the three fractions. T.l.c. of the three fractions from labelled urine showed the same labelled materials in each fraction. For further purification only the ammoniacal fraction was used. This fraction contained most of the radioactivity and was not grossly contaminated with inorganic material.

Purification by t.l.c. The ammoniacal fraction from the Florisil column was freeze-dried, dissolved in approx. 2 ml of water and centrifuged. Any solid material was washed

twice with a small volume of water, the washings were combined with the original supernatant and further purified by t.l.c. as follows.

The solution was applied as a streak (with a SA20 streak applicator; Burkard, Rickmansworth, Herts., U.K.) to cellulose t.l.c. plates (200 mm × 200 mm × 1 mm). Further purification was on plates 1 mm or 250 μm thick. All chromatograms were developed to 10 cm from the origin. Recovery of label ([2-¹⁴C]PteGlu) from cellulose plates was 70%.

Because of the anomalous behaviour of small amounts of folic acid (Blair & Dransfield, 1969) in solvent (a), 2 mg of non-radioactive PteGlu (*R_f* 0.00) was added as a routine to the Florisil ammoniacal fraction before chromatography. It was also found that this acidic solvent system caused retention of radioactive material at the origin; subsequent chromatography in systems (b) and (c) showed that this material was not PteGlu. This retention may be due to washings from the Florisil column. To avoid erroneous conclusions due to this retention, chromatograms were also run in the alkaline solvent (c), which did not cause retention at the origin.

Radioautography. This was used for the detection and location of labelled compounds on chromatograms. An X-ray film (Ilford Industrial G, Ilford, Essex, U.K.) was placed over the chromatogram and held in position by a further glass plate and elastic bands. X-ray films were exposed at –15°C for up to 21 days, developed in Kodak DX 80, washed in 3% (v/v) acetic acid and fixed in Kodak FX 40. Minimum detection of radioactivity by this technique was approx. 2.5 nCi/cm² in 24 h. The relative amounts of labelled compounds were estimated by scanning the radioautograph with a microdensitometer (MK IIIc; Joyce, Lobel and Co. Ltd., Gateshead, Co. Durham, U.K.).

Microbiological assays. Samples were dissolved in 0.1 M-sodium phosphate buffer, pH 7.0, containing 1% (w/v) ascorbic acid. The organisms used were *Lactobacillus casei* N.C.I.B. 8010 grown in Q-Ess medium (gift of BBL, Division of Bioquest, U.S.A.), *Streptococcus faecalis* N.C.I.B. 8043, grown in Difco 0319-15 medium and *Pediococcus cerevisiae* N.C.I.B. 7837 grown in Difco 0456-15 medium (Difco Laboratories, Detroit, Mich., U.S.A.). Incubation was for 18 h at 37°C and the extent of growth was determined turbidimetrically. Assays were performed in duplicate at least. Chicken pancreas conjugase was prepared by the method of Perry & Chanarin (1968).

Reduction of labelled compounds. This was done with excess of NaBH₄ in 2% (w/v) ammonium acetate, pH 7.0, containing 0.5% (v/v) 2-mercaptoethanol, for 20 min at room temperature, or by catalytic hydrogenation in acetic acid, H₂/Pt, for 20 min at room temperature.

Formylation of 5-methyltetrahydropteroyl-L-glutamic acid. Formic acid (98%, w/v) reacts with H₄PteGlu to produce 10-CHO-H₄PteGlu (May *et al.* 1951), which rearranges in acidic solutions to the more stable 5,10-CH=H₄PteGlu (May *et al.* 1951). A similar overall reaction has been carried out in this laboratory by using 5-CH₃-H₄PteGlu. The 5-CH₃-H₄PteGlu (6 mg) was dissolved in 98% formic acid (2 ml) containing 1% (w/v) ascorbic acid and covered with a layer of toluene. After 3 h the mixture was purified by t.l.c. on cellulose. The major fluorescent material (white; under 365 nm u.v. light), *R_f* 0.35 in solvent (a), was identified as 5,10-CH=H₄PteGlu by fluorescence

spectrum (excitation maximum 335nm; emission maximum 470nm), u.v. spectrum (peak at 350nm in 0.1M-HCl disappears when the solution is made alkaline and reappears on acidification) and t.l.c. on cellulose [R_F 0.36 in solvent (e), 0.34 in solvent (f), 0.38 in solvent (g), 0.22 in solvent (h) and 0.06 in solvent (i)]. Authentic 5,10-CH₂=H₄PteGlu (calcium leucovorin dissolved in 0.1M-HCl) had R_F values 0.35, 0.34, 0.39, 0.21 and 0.05 in these solvents respectively and co-chromatographed with the material in solvent systems (a) and (h).

The reaction with 5-CH₃-H₄PteGlu was slower than with H₄PteGlu. The reaction was also carried out at 75°C and with aq. 10% formic acid at room temperature.

RESULTS

The urinary excretion of radioactivity after an oral dose of [2-¹⁴C]PteGlu is shown in Table 2.

Identification of the labelled urinary components was attempted by purification of urine collected under various conditions as follows.

Collection of urine without antioxidants. When urine was collected in either acid or alkali without antioxidants present, extensive and variable decomposition of radioactive material to PteGlu occurred, during both collection and purification procedures; consequently all subsequent experiments were performed with added antioxidants (see the Methods section).

Collection of urine in acid with use of antioxidants throughout. Typical chromatograms of the ammoniacal Florisil fraction showed radioactivity at R_F values (0.00, due to anomalous retention), 0.28, 0.42 and 0.52 in solvent (a) and corresponding R_F values 0.25, 0.10 and 0.40 in solvent (c). Labelled PteGlu was absent. The compound with R_F 0.52 in solvent (a) and 0.40 in solvent (c) was identified as 5-CH₃-H₄PteGlu by co-chromatography in solvent systems (a), (b), (c) and (j).

Radioactive compounds were purified free from any fluorescence or u.v. absorption by chromatography in solvent (a), extraction, freeze-drying and repetition of this process in solvents (c) and (d). The amounts of radioactive compounds were too small to be detected under u.v. light by using this method.

Collection of the urine frozen followed by freeze-drying. Antioxidants were used during purification. Chromatography in solvent (a) showed the presence of five labelled compounds with R_F values (0.00), 0.22, 0.31, 0.43 (major radioactivity) and 0.52 (identified as 5-CH₃-H₄PteGlu). Labelled PteGlu was absent. The major radioactivity was further purified in solvent (c) and had R_F 0.10, and then in solvent (d), in which it had R_F 0.47, and was then free from all fluorescence or u.v. absorption.

Labelled 5-CH₃-H₄PteGlu was identified (by its R_F value in two solvents) by a similar purification in all but one of 12 subsequent experiments. Characterization by co-chromatography in three solvents was carried out in eight of these experiments.

The relative microbiological activity of the major unidentified compound, R_F 0.42 in system (a) and 0.10 in system (c), was 100 for *L. casei*, 13–38 (mean 25) for *S. faecalis* and <2.5 for *P. cerevisiae*. The corresponding values for the isolated 5-CH₃-H₄[2-¹⁴C]PteGlu were 100, 4–33 (mean 12) and <2.5. *L. casei* assays on the unidentified material before and after conjugase treatment were exactly the same indicating that this compound is not a heptaglutamate. The response of *S. faecalis* and *P. cerevisiae* to conjugase-treated material was not determined.

Collection of urines with added folates. Because of the instability of the unidentified compound and its decomposition to PteGlu, even when antioxidant was present, we decided to investigate the identity of this compound by the addition of folates to the urine collection flask, when, even if decomposition did occur, the radioactive material would correspond to a fluorescent pteridine formed from the decomposed folate.

When urine was collected in the presence of added PteGlu (0.5mg), H₂PteGlu (1mg), H₄PteGlu (5mg), 5-HCO-H₄PteGlu (0.5mg dissolved in 1M-HCl), 5,10-CH₂-H₄PteGlu (2mg), in separate experiments, the radioactive compounds did not correspond to the fluorescence or u.v. absorption of the added folate or, in the case of H₄-PteGlu and

Table 2. Urinary excretion of radioactivity after an oral dose of [2-¹⁴C]pteroyl-L-glutamic acid

The results are expressed as the percentage of the administered radioactivity (mean $\pm$ s.d.; no. of determinations in parentheses) excreted in the urine after oral doses of [2-¹⁴C]PteGlu, specific radioactivity 55.3 mCi/mmol.

Dose (μ g/kg)	Percentage excreted in urine		
	0–24 h (1st day)	24–48 h (2nd day)	48–72 h (3rd day)
3.12	6.83 $\pm$ 2.91 (12)	2.66 $\pm$ 0.78 (4)	—
32.0	5.89 $\pm$ 2.56 (8)	1.95 $\pm$ 0.83 (8)	1.05 $\pm$ 0.77 (8)
320	30.7 $\pm$ 6.8 (14)	1.22 $\pm$ 0.18 (4)	0.64 $\pm$ 0.29 (4)

5,10-CH₂-H₄PteGlu, which were not themselves isolated, of their decomposition products. Added 5-CH₃-H₄PteGlu did not alter the radioactive materials isolated.

Reduction of the unidentified compound. The attempted reduction of the labelled material (purified through Florisil and by t.l.c. in two solvent systems) with sodium borohydride and by catalytic hydrogenation, did not change the pattern of radioactive spots when compared with control experiments.

Purification of the second-day urines. This was performed in a manner exactly similar to that used for the first-day urines, antioxidants being used throughout. The radioautographs were essentially similar to those of the chromatograms produced by the first-day urines. Radioactive PteGlu was absent. The major radioactive component, R_F 0.48 in solvent (a) and 0.11 in solvent (c), was not identified. The compound with R_F 0.55 in solvent (a) was identified as 5-CH₃-H₄PteGlu by co-chromatography in solvent (b), R_F 0.83, and solvent (c), R_F 0.41.

Purification of the third-day urines. This was performed with use of antioxidants throughout. The radioautograph showed the presence of similar radioactive compounds and labelled 5-CH₃-H₄-PteGlu was identified by its R_F value in solvent systems (a) and (c).

Studies on the two major metabolites in the urine. The ratio of the major unidentified material to the 5-CH₃-H₄[2-¹⁴C]PteGlu, isolated on the first day after a dose of 32 µg/kg, varied from 0.5–7.7 (mean 2.7) to 1.0 in twelve experiments. The percentage of the total radioactivity on the chromatograms in the unidentified material and 5-CH₃-H₄PteGlu, and the specific radioactivity of the 5-CH₃-H₄-PteGlu, are shown in Table 3.

The specific radioactivity of the unidentified material, assuming full activity for *L. casei*, isolated on the first day after an oral dose of 32 µg/kg, was

2.15 mCi/g of PteGlu, giving a dilution of the starting material of 59.1. This was similar to the dilution (52.2) of the 5-CH₃-H₄PteGlu.

The results of a series of orally administered 'flushing' doses of unlabelled PteGlu are shown in Table 4. Flushing doses of up to 1.6 mg/kg body wt., given at 24 h or later after oral doses of 3.95 or 32 µg of labelled PteGlu/kg, did not produce a significant increase in radioactivity in the urine, and did not give labelled PteGlu in the urine. The percentage of the radioactivity in the urine present as 5-CH₃-H₄PteGlu was 9.5 after a flushing dose of 200 µg/kg given 72 h later, 26 (specific radioactivity 0.013 mCi/mmol) after a flushing dose of 400 µg/kg given 24 h later, and 30 (specific radioactivity 0.008 mCi/mmol) after a flushing dose of 400 µg/kg given 72 h later.

A significant increase (Student's *t* test, *P* < 0.001) in radioactivity occurred when doses of 1.6 mg of unlabelled PteGlu/kg were given 3.5 h after or 3.5 h before the labelled dose (44 µg/kg). Labelled PteGlu was excreted and represented 45% and 49% respectively of the radioactivity detected on the chromatograms.

Tissue radioactivity. The radioactivity present in the faeces, urine, liver and kidney after oral doses of [2-¹⁴C]PteGlu is shown in Table 5.

DISCUSSION

The experimental results show that of a dose of 80 µg (320 µg/kg) only 20–40% of the PteGlu was absorbed, and of this 81% was excreted in the urine in 7 days. At the PteGlu dose (5.5 µg) equivalent to the physiological range of folate (approx. 5 µg per day), 50% was absorbed and of that 31% was excreted in 6 days. Although the faecal excretion of radioactivity was not determined for a dose of 0.8 µg, it seems likely that about 50% was absorbed, by comparison of the tissue contents with those after a dose of 5.5 µg. A similar amount of excretion

Table 3. *Studies on the isolated 5-CH₃-[2-¹⁴C]H₄PteGlu*

After an oral dose of [2-¹⁴C]PteGlu (specific radioactivity 55.3 mCi/mmol) the urine was purified by column chromatography and t.l.c. The percentages of the radioactivity on the chromatograms present as the unidentified compound and as 5-CH₃-H₄PteGlu were estimated by densitometry of radioautographs and the specific radioactivity of 5-CH₃-H₄PteGlu by a combination of liquid-scintillation counting and *L. casei* assay, on material eluted from thin-layer chromatograms.

Dose (µg/kg)	Day	Radioactivity (%) present as		Sp. radioactivity of 5-CH ₃ -H ₄ [2- ¹⁴ C]- PteGlu (mCi/mmol)
		Unidentified compound	5-CH ₃ -H ₄ PteGlu	
2.2	1	60	22	0.097
32.0	1	43.3	29	1.06
	2	82.6	14	0.032
	3	67.0	8.6	—
320	1	52	27	17.1

Table 4. *Displacement of radioactivity by flushing doses*

Flushing doses of PteGlu were given by dissolving the PteGlu in a minimum volume of aq. 1% (v/v) NH_3 , diluting it with water (final pH 7–8) and administering in 0.5 ml by stomach intubation. The results are expressed as the percentage of the administered radioactivity (mean $\pm$ s.d. of four animals) excreted in the 24 h before and in the days after an oral flushing dose of unlabelled PteGlu. The specific radioactivity of the $[2\text{-}^{14}\text{C}]\text{PteGlu}$ was 55.3 mCi/mmol.

Dose of $[2\text{-}^{14}\text{C}]\text{-PteGlu}$ ($\mu\text{g/kg}$)	Time (h) of the flushing dose after the radioactive dose	Flushing dose (μg of PteGlu/kg)	% of the administered radioactivity appearing in the urine			
			24 h before	Days after flushing dose		
				1	2	3
32.0	72	200	1.72 ± 0.23	2.07 ± 0.99	1.02 ± 1.14	0.62 ± 0.09
35.2	72	400	0.39 ± 0.40	$1.00 \pm 0.20^*$	0.68 ± 0.12	0.33 ± 0.14
32.0	24	400	12.1 ± 8.3	$2.93 \pm 0.56^\dagger$	1.35 ± 0.55	1.21 ± 0.51
3.95	24	400	6.87 ± 4.40	4.71 ± 1.30	—	—
21.8	24	1600	4.27 ± 2.23	2.44 ± 0.87	1.22 ± 0.27	—
44.3	3.5	1600	=	16.5 ± 0.8	1.12 ± 0.28	1.12 ± 0.24
43.6	Preloading dose of 1600 μg of PteGlu/kg given 3.5 h before the labelled dose		=	20.2 ± 4.6	0.79 ± 0.30	0.68 ± 0.42

* Specific radioactivity of the 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ was 0.008 mCi/mmol.

† Specific radioactivity of the 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ was 0.013 mCi/mmol.

Table 5. *Distribution of radioactivity after oral doses of $[2\text{-}^{14}\text{C}]\text{PteGlu}$*

The radioactivity in the urine (collected every 24 h after administration of $[2\text{-}^{14}\text{C}]\text{PteGlu}$) was assayed directly by liquid-scintillation spectrometry. The radioactivity in the liver, kidney and faeces (collected throughout the experiment) was assayed by combustion followed by liquid-scintillation counting (see the Methods section). The specific radioactivity of the $[2\text{-}^{14}\text{C}]\text{PteGlu}$ was 55.3 mCi/mmol. Results are the mean $\pm$ s.d. for four animals.

Dose ($\mu\text{g/kg}$)	Duration of experiment (days)	Radioactivity (% of oral dose)			
		Faeces	Urine	Liver	Kidney
320	7	74.9 ± 33.1	32.6 ± 4.5	7.3 ± 2.0	0.56 ± 0.12
22	6	47.6 ± 4.7	12.0 ± 3.0	16.1 ± 2.8	1.1 ± 0.3
3.2	3	—	10.9 ± 3.7	14.4 ± 3.7	2.2 ± 0.3

was observed in the rat by using *S. faecalis* assays (Swendseid, Bird, Brown & Bethell, 1947). During studies on humans, Retief (1969), using *L. casei* assays, obtained similar results, whereas higher values were recorded by Jukes, Franklin, Stokstad & Boehne (1947) using *L. casei*, by Girdwood & Delamore (1961) using *S. faecalis* assays and by Anderson *et al.* (1960) using tritiated PteGlu. Wide variations of excretion have been recorded and may reflect differences in folate absorption and metabolism, in the microbiological activity of isolated materials and in the specific radioactivity when labelled PteGlu was used. The amounts of label remaining in the liver were approximately an order of magnitude greater than those in the kidney at all three doses and are within the ratios reported by using *L. casei* assays (Grossowicz, Rachmilewitz & Izak, 1963; Grossowicz, Izak & Rachmilewitz, 1964).

There was no significant difference in the per-

centage of the administered radioactivity (approx. 6%) appearing in the urine in the 24 h period after oral doses of 3.2 and 32 $\mu\text{g/kg}$ of $[2\text{-}^{14}\text{C}]\text{PteGlu}$, but a marked increase (to 30%) when the dose was raised to 320 $\mu\text{g/kg}$. On the second and third days, the urinary excretion of radioactivity after the largest dose fell sharply, whereas that after the smaller doses fell more gradually. This pattern of urinary excretion is in agreement with those found by Steinkamp, Shukers, Totter & Day (1946), Jukes *et al.* (1947), Denko (1951) and Girdwood & Delamore (1961), in that the largest amount of the excreted material from an oral dose is excreted within 24 h.

After an oral dose of 32 $\mu\text{g/kg}$ collection in acid and alkali and purification of the first-day urine without antioxidants led to decomposition and only PteGlu was identified. A similar purification procedure with antioxidants present led to the isolation

of tetrahydropteroylglutamates, and PteGlu was absent. Similar results were obtained by collection in acid, alkali and with frozen urine. PteGlu, isolated by several workers (Kinnear *et al.* 1963; Anderson *et al.* 1960; Johns *et al.* 1961, using [^3H]PteGlu and DEAE-cellulose purification; and Baker *et al.* 1965, using bioautography), probably arose by oxidation due to the absence of antioxidants as in our work. McLean & Chanarin (1966), using antioxidants, also obtained PteGlu. Specific radioactivities, which would indicate whether or not decomposition had taken place, were not recorded by the authors who used labelled material.

During the present work two major (representing some 85% of the radioactivity) and one minor labelled metabolites were isolated. One of the major metabolites was identified as 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$. The other was not identified but was not PteGlu, 7,8- H_2PteGlu , H_4PteGlu , 5- $\text{HCO-H}_4\text{PteGlu}$, 5,10- $\text{CH=H}_4\text{PteGlu}$, 10- $\text{HCO-H}_4\text{PteGlu}$, 5- $\text{HCNH-H}_4\text{PteGlu}$, 5,10- $\text{CH}_2\text{-H}_4\text{PteGlu}$ or 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$, nor any artifact formed from these during the isolation. Labelled unconjugated pteridines were not isolated, although they were not extensively studied because their R_F values differed from the radioactive materials and the radioactive compounds decomposed to PteGlu when antioxidants were absent. 2-Amino-4-hydroxypteridine and 10-formylpteroylglutamic acid appeared as extremely small amounts of radioactivity on only two occasions and they were derived from other labelled materials.

The microbiological activity of the 5- $\text{CH}_3\text{-H}_4\text{-PteGlu}$ showed maximum activity for *L. casei* and little activity for *S. faecalis*, which was nevertheless higher than expected and may have been caused by decomposition during the assay. The unidentified material showed similar specific radioactivity to the 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ and therefore was a naturally occurring metabolite of PteGlu. Its activity with *L. casei* was identical before and after conjugase treatment; the molecule therefore is unlikely to contain more than three glutamic acid residues. The compound was not reducible. From this evidence and the R_F values, it is tentatively identified as the di- or tri-glutamate of 5-methyltetrahydropteroate. Because of the similarity in microbiological activity these methods cannot distinguish between the unidentified compound and 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$.

Early work suggested an increase in *P. cerevisiae* activity (citrovorum factor) in the urine after oral administration of PteGlu but isolation media often contained no antioxidants and compounds with citrovorum factor activity were accompanied by unidentified *S. faecalis*-active material. To account for the citrovorum factor activity, first reported by Sauberlich (1949), two groups of workers (Albrecht

& Broquist, 1956; Silverman *et al.* 1956) proposed that it was 5,10- $\text{CH=H}_4\text{PteGlu}$, which was isolated by Silverman *et al.* (1956). These results must be viewed with caution, since both groups used very large doses (up to 175 mg for humans) and Silverman *et al.* (1956) used formic acid, which, we have shown, reacts with 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ to give 5,10- $\text{CH=H}_4\text{-PteGlu}$. Low conversions into urinary citrovorum factor activity have been reported for PteGlu when given orally (Anderson *et al.* 1960; Broquist, Stokstad & Jukes, 1951; Baker *et al.* 1965; Oji, Wada & Yoshida, 1951) or subcutaneously (Guggenheim, Halevy, Neumann & Usiel, 1956). More recently McLean & Chanarin (1966) have shown no activity for *P. cerevisiae*. We did not obtain 5,10- $\text{CH=H}_4\text{PteGlu}$ and the compounds isolated showed no activity for *P. cerevisiae*. Comparable observations have been reported for blood; Usdin (1959), using no antioxidants, obtained 5- $\text{HCO-H}_4\text{PteGlu}$ (active for *P. cerevisiae*) although 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ was the major folate present.

Labelled unconjugated pteridines were not present in the urine. Neopterin (Sakurai & Goto, 1967) and biopterin (Broquist & Albrecht, 1955; Patterson *et al.* 1955, 1956) may have been derived from compounds in the diet or by synthesis from purines (reviewed by Kaufman, 1967), although this has not been shown for mammals. Isoxanthopterin (Blair, 1958) may have been derived by folate degradation. Orally, subcutaneously or intravenously administered PteGlu did not give rise to increased xanthopterin in the urine (Rauen & Haller, 1950); the origin of xanthopterin (Koschka & Hrubesch, 1939) is uncertain.

The 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ and the unidentified material were also obtained on the second and third days of excretion, and also at oral doses of 3.2 and 320 $\mu\text{g/kg}$. The specific radioactivity of the 5- $\text{CH}_3\text{-H}_4\text{PteGlu}$ isolated from day 1 samples at the three dosages showed that this metabolite had been diluted by 100–200 μg of folate, which is about half the total hepatic folate found by using *L. casei* (Bird, McGlohon & Vaitkus, 1965).

A large dose (1.6 mg/kg) of non-labelled PteGlu given orally 3.5 h before or 3.5 h after [^{14}C]PteGlu produced an increase in radioactivity and labelled PteGlu in the urine. This is in agreement with absorption of PteGlu without metabolism (Whitehead & Cooper, 1967; Butterworth, Baugh & Krumdieck, 1969; Smith, Matty & Blair, 1970), but in disagreement with Cohen (1967) who obtained metabolites on the serosal side of everted-sac preparations, although the possibility that these arose from endogenous folate was not ruled out. Chanarin & Perry (1969) obtained an increase in growth-promoting activity for *L. casei* but no increase in activity for *S. faecalis* (which grows with H_2PteGlu and H_4PteGlu) in the systemic blood

after oral doses of tritiated $H_2PteGlu$ and $H_4PteGlu$, and concluded that metabolism to 5- CH_3 - $H_4PteGlu$ occurred during absorption. However, the peak of radioactivity occurred significantly earlier than the peak of *L. casei* activity and the inability of these investigators to measure portal-blood folate also does not warrant their conclusion (Bernstein, Gutstein, Weiner & Efron, 1970).

Flushing doses of unlabelled $PteGlu$ given 24 h or more after the $[2-^{14}C]PteGlu$ produced no significant increase in radioactivity excreted. Similar results were obtained for humans (Butterworth *et al.* 1969). The small amounts of radioactivity were accompanied by low specific radioactivity of the metabolites, showing extensive dilution owing to metabolism of the flushing dose. Labelled $PteGlu$ was not present in the urine, which suggests that the $[2-^{14}C]PteGlu$ had been completely metabolized within 24 h. This was substantiated in that labelled $PteGlu$ was not present in the liver 24 h after an oral dose of 320 $\mu g/kg$ (J. A. Blair & E. Dransfield, unpublished work). These results are in disagreement with those of Johns *et al.* (1961) who obtained displacement of tritiated $PteGlu$ by intravenous flushing doses. They did not use antioxidants and their results are subject to criticism on the grounds of decomposition of metabolites and the displacement of radioactivity found by them, but not by ourselves nor by Butterworth *et al.* (1969), may reflect the high specific radioactivity of the tritiated $PteGlu$.

Metabolic studies can now be undertaken by using the biologically active diastereoisomers isolated during this work.

We are grateful to Mr R. J. Leeming and Miss H. Portman-Graham for the microbiological assays, and to the Wellcome Trust for financial support.

REFERENCES

- Albrecht, A. M. & Broquist, H. P. (1956). *Proc. Soc. exp. Biol. Med.* **92**, 158.
- Anderson, B., Belcher, E. H., Chanarin, I. & Mollin, D. L. (1960). *Br. J. Haemat.* **6**, 439.
- Baker, H., Frank, O., Feingold, S., Ziffer, H., Gellene, R. A., Leevy, C. M. & Sobotka, H. (1965). *Am. J. clin. Nutr.* **17**, 88.
- Bernstein, L. H., Gutstein, S., Weiner, S. & Efron, G. (1970). *Am. J. Med.* **48**, 570.
- Bird, O. D., McGlohon, V. M. & Vaitkus, J. W. (1965). *Analyt. Biochem.* **12**, 18.
- Blair, J. A. (1958). *Biochem. J.* **68**, 385.
- Blair, J. A. & Dransfield, E. (1969). *J. Chromat.* **45**, 476.
- Blair, J. A. & Saunders, K. J. (1970). *Analyt. Biochem.* **34**, 376.
- Blakley, R. L. (1969). *The Biochemistry of Folic Acid and Related Pteridines*, p. 29. Amsterdam and London: North-Holland Publishing Co.
- Broquist, H. P. & Albrecht, A. M. (1955). *Proc. Soc. exp. Biol. Med.* **89**, 178.
- Broquist, H. P., Stokstad, E. L. R. & Jukes, T. H. (1951). *J. Lab. clin. Med.* **38**, 95.
- Burgen, A. S. V. (1961). *Blood*, **17**, 367.
- Butterworth, C. E., Baugh, C. M. & Krundieck, C. (1969). *J. clin. Invest.* **48**, 1131.
- Chanarin, I. & Perry, J. (1969). *Lancet*, ii, 776.
- Cohen, N. (1967). *Clin. Res.* **13**, 257.
- Decastello, A. (1935). *Medsche. Klin.* **31**, 377.
- Denko, C. W. (1951). *J. appl. Physiol.* **3**, 559.
- Futtermann, S. (1957). *J. biol. Chem.* **228**, 1031.
- Girdwood, R. H. & Delamore, I. W. (1961). *Scott. Med. J.* **6**, 44.
- Grossowicz, N., Izak, G. & Rachmilewitz, M. (1964). *Proc. Soc. exp. Biol. Med.* **115**, 953.
- Grossowicz, N., Rachmilewitz, M. & Izak, G. (1963). *Proc. Soc. exp. Biol. Med.* **112**, 486.
- Guggenheim, K., Halevy, S., Neumann, H. & Usiel, V. (1956). *Biochem. J.* **62**, 281.
- Hatefi, Y., Talbert, P. T., Osborn, M. J. & Huennekens, F. M. (1960). *Biochem. Prep.* **7**, 89.
- Johns, D. G., Sperti, S. & Burgen, A. S. V. (1961). *J. clin. Invest.* **40**, 1684.
- Jukes, T. H., Franklin, A. L., Stokstad, E. L. R. & Boehne, J. W. (1947). *J. Lab. clin. Med.* **32**, 1350.
- Kalberer, F. & Rutschmann, J. (1961). *Helv. chim. Acta*, **44**, 1956.
- Kaufman, S. (1967). *A. Rev. Biochem.* **36**, 171.
- Kinnear, D. G., Johns, D. G., MacIntosh, P. C., Burgen, A. S. V. & Cameron, D. G. (1963). *Can. med. Ass. J.* **89**, 975.
- Koschara, W. & Hrubesch, A. (1939). *Hoppe-Seyler's Z. physiol. Chem.* **258**, 39.
- McLean, A. & Chanarin, I. (1966). *Blood*, **27**, 386.
- May, M., Bardos, T. J., Barger, F. L., Lansford, M., Ravel, J. M., Sutherland, G. L. & Shive, W. (1951). *J. Am. chem. Soc.* **73**, 3067.
- Oji, K., Wada, M. & Yoshida, T. (1951). *Med. J. Osaka Univ.* **5**, 177.
- Patterson, E. L., Broquist, H. P., Albrecht, A. M., Saltza, M. H. & Stokstad, E. L. R. (1955). *J. Am. chem. Soc.* **77**, 3167.
- Patterson, E. L., Saltza, M. H. & Stokstad, E. L. R. (1956). *J. Am. chem. Soc.* **78**, 5871.
- Perry, J. & Chanarin, I. (1968). *Br. med. J.* iv, 546.
- Rauen, H. M. & Haller, V. H. (1950). *Hoppe-Seyler's Z. physiol. Chem.* **286**, 96.
- Retief, F. P. (1969). *Br. med. J.* i, 150.
- Sakurai, A. & Goto, M. (1967). *J. Biochem., Tokyo*, **61**, 142.
- Sauberlich, H. E. (1949). *J. biol. Chem.* **181**, 467.
- Silverman, M., Ebaugh, F. G. & Gardiner, R. C. (1956). *J. biol. Chem.* **223**, 259.
- Smith, M. E., Matty, A. J. & Blair, J. A. (1970). *Biochim. biophys. Acta*, **219**, 37.
- Steinkamp, R., Shukers, C. F., Totter, J. R. & Day, P. L. (1946). *Proc. Soc. exp. Biol. Med.* **63**, 556.
- Swendseid, M. E., Bird, O. D., Brown, R. A. & Bethell, F. M. (1947). *J. Lab. clin. Med.* **32**, 23.
- Usdin, E. (1959). *J. biol. Chem.* **234**, 2373.
- Whitehead, V. M. & Cooper, B. A. (1967). *Br. med. J.* xiii, 679.
- Wieland, O. P., Hutchings, B. L. & Williams, J. H. (1952). *Archs Biochem. Biophys.* **40**, 205.
- Zakrzewski, S. F., Evans, E. A. & Phillips, R. F. (1970). *Analyt. Biochem.* **36**, 197.