Journal of Chromatography, 79 (1973) 195–207
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CHROM. 6597

THIN-LAYER CHROMATOGRAPHY OF PTEROYLGLUTAMATES AND RELATED COMPOUNDS*

APPLICATION TO TRANSPORT AND METABOLISM OF REDUCED FOLATES IN BLOOD

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(First received November 6th, 1972; revised manuscript received January 15th, 1973)

SUMMARY

A thin-layer chromatographic (TLC) system comprised of cellulose powder (MN 300 UV_{254}) and 3.0 % (w/v) NH_4Cl and 0.5 % (v/v) 2-mercaptoethanol gives improved resolution of pteroylmonoglutamates and several related compounds. In addition to resolving pteroylglutamate, dihydropteroylglutamate and tetrahydropteroylglutamate, previously achieved by an ion-exchange TLC system, the three formylated tetrahydropteroylglutamates 5-formyl, 10-formyl and 5, 10-methenyl (5-CHO-H_4-PteGlu, 10-CHO-H_4-PteGlu, and 5,10-CH=H_4-PteGlu, respectively) are also resolved with mean R_F values of 0.73, 0.82 and 0.32, respectively. The reduced 5-methyl (5-CH_3-H_4-PteGlu) and 10-formyl derivatives are not adequately separated but may be distinguished by conversion of the formylated derivative to the 5,10-methenyl derivative by acidification or by microbiological assay. Similarly, while the reduced 5-formyl and 5-formimino (5-HCNH-H_4-PteGlu) derivatives exhibit identical R_F values, by conversion of the former to the 5,10-methenyl derivative they can be resolved. Separation of 5-CHO-H_4-PteGlu and 5-HCNH-H_4-PteGlu has not been possible. The chromatographic behaviour of various other folate derivatives and degradation products was also determined.

Transport and fate of labelled derivatives were studied in heparinized blood. [3′,5′,9-$^3H_3$]PteGlu, 5-[^{14}C]CH_3-H_4-PteGlu and H_4[6,7-$^3H_2$]PteGlu appeared to be well incorporated (6–10 %, 12–24 h), whereas H_2-[3′,5′,9-$^3H_3$]PteGlu and 5-CHO-H_4-[6,7-$^3H_2$]PteGlu were not. Lysates of cells incubated with PteGlu or 5-CH_3-PteGlu (^3H- or ^{14}C-labelled) when analyzed by TLC (in conjunction with γ-glutamyl carboxypeptidase treatment) revealed that neither compound was metabolized up to 12 h after incorporation.

INTRODUCTION

The rapid and convenient separation of naturally occurring folates (pteroylmonoglutamates) is essential to investigations of their distribution and metabolism.

* A preliminary report of this work was presented at the 7th Meeting of the Federated European Biochemical Societies, Varna, Bulgaria, 1971.

This communication describes a thin-layer chromatographic (TLC) system which appears to surpass those previously described both in simplicity and overall resolution for pteroylmonoglutamates and several related compounds.

A small amount of information is available concerning the extent to which pteroylglutamate is taken up by blood cells[1–3], however, whether reduced folates are taken up has not been studied. In addition, the subsequent fate of any form of folate after it has been removed by the blood cells remains unknown. Several radioactive folate derivatives were employed *in vitro* to assess their transport and possible subsequent metabolism.

EXPERIMENTAL

Materials and TLC methods

Materials for TLC were obtained from the following sources: thin-layer applicator and chromatographic chambers from, Desaga, Heidelberg, G.F.R.; cellulose powder MN 300 UV_{254} from Macherey, Nagel & Co., Düren, G.F.R.; Bio-Rad analytical-grade cation-exchange resins (AG50W-X4 and AG50W-X8, 200–400 mesh), anion-exchange cellulose for TLC (Cellex D), and cation-exchange cellulose for TLC (Cellex CM) from Micro-Bio Laboratories Ltd., London, Great Britain. Cellulose layers were applied in methanol to glass plates (20 × 20 cm and 20 × 5 cm) in thicknesses of 0.25 and 0.50 mm and were allowed to air dry.

Numerous solvent systems were investigated with the MN 300 UV_{254} cellulose powder, among them: 3.0 % NH_4Cl; (15 %w/v) Na_2HPO_4; 0.1 *M* sodium phosphate buffers, pH 6.0 and pH 7.0; *n*-propanol–water–0.88 sp.gr, aqueous ammonia (20:10:1); *n*-butanol–glacial acetic acid–water (4:1:5), upper phase; 5 % citrate–ammonium hydroxide–isoamyl alcohol saturated, pH 9.0; *n*-butanol–water-ethanol–glacial acetic acid (52:28:20:0.3); 5 % acetic acid; isopropanol–pyridine–water (1:1:1); 5 % NH_4HCO_3; 1 *M* ammonium hydroxide–ethanol (1:1); 2 % NaCl; *sec.*-butanol-formic acid–water (8:2:5); glycine buffer, pH 9.5, containing 0.29 % disodium ethylenediaminetetraacetate; 1 *M* sodium formate with 2 % formic acid. All of the solvents contained 0.5 % (v/v) 2-mercaptoethanol (MET) as antioxidant.

In addition, cation-exchange TLC plates were prepared with Whatman CC41 and MN 300 UV_{254} cellulose powders and AG50W-X4 and AG50W-X8 resins according to the recommendations of COPENHAVER AND O'BRIEN[4].

Mixtures (1:4, w/w) of the ion-exchange celluloses Cellex CM, Cellex D, and the MN 300 UV_{254} powder were also used. Compounds were usually visualized under ultraviolet (UV) light at 254 or 366 nm. The R_F values were obtained by dividing the distance from the origin to the centre of the visualized compound by the distance the solvent travelled, which was usually 150 to 160 mm.

Pteroylmonoglutamates

Pteroylmonoglutamates and related compounds were obtained from the following sources: pteroyl-L-glutamic acid (PteGlu) from Cyanamid of Great Britain Ltd., Gosport, Hants., Great Britain; methotrexate (4-NH_2-10-CH_3-PteGlu) and Calcium Leucovorin (5-CHO-H_4-PteGlu) were gifts of Lederle Division American Cyanamid Co., Pearl River, N.Y., U.S.A.; *p*-aminobenzoyl-L-glutamic acid (PABGlu) and L-glutamic acid (Glu) from Sigma Chemical Co. Ltd., London, Great Britain; benzoic

acid (BA) from May & Baker Ltd., Dagenham, Great Britain; *p*-aminohippuric acid and *p*-aminobenzoic acid (PABA) from Koch-Light Laboratories Ltd., Bucks., Great Britain; pterin (Pt or 2-NH_2-4-OH-pteridine) from Fluka AG, Buchs SG, Switzerland; pterin-6-carboxylic acid (Pt-6-COOH), *p*-acetamidobenzoic acid, 2-mercaptopteridine, isoxanthopterin (Pt-7-OH), and pterin-7-carboxylic acid (Pt-7-COOH) from Aldrich Chemical Co., Milwaukee, Wisc., U.S.A.; xanthopterin (Pt-6-OH) and 7-methyl, xanthopterin (7-CH_3-Pt-6-OH) from Dr. Theodor Schuchardt GmbH. & Co., Hohenbrunn, G.F.R.; 4-hydroxypteridine and lumazine (2,4-dihydroxypteridine) from Ralph Emanuel Ltd., Middlesex, Great Britain; L-biopterin, 6-(L-*erythro*-1,2-dihydroxypropyl)pterin, L-neopterin, and 6-(L-*erythro*-1,2,3-trihydroxypropyl)pterin were gifts of Roche Products Ltd., Welwyn Garden City, Herts., Great Britain; *l*-5-formimino-tetrahydropteroylglutamate (5-HCNH-H_4-PteGlu) was a gift of Dr. H. UYEDA[5].

Several compounds were prepared due to their unavailability or instability. Calcium leucovorin was dissolved in 0.1 *M* HCl to obtain 5,10-methenyl-tetrahydropteroylglutamic acid (5,10-CH=H_4-PteGlu) and upon neutralization of the latter 10-formyl-tetrahydropteroylglutamic acid (10-CHO-H_4-PteGlu) was also obtained. Tetrahydropteroylglutamic acid (H_4-PteGlu) was prepared from PteGlu both by borohydride reduction[6] and catalytic hydrogenation with PtO_2. 5-Methyl-tetrahydropteroylglutamate (5-CH_3-H_4-PteGlu) barium and calcium salts were prepared by the method of BLAIR AND SAUNDERS[7]. Dihydropteroylglutamic acid (H_2-PteGlu) was prepared by dithionite reduction of PteGlu[8,15] and stored as a suspension in 0.005 *M* HCl. 5,10-Methylene-tetrahydropteroylglutamic acid (5,10-CH_2-H_4-PteGlu) was prepared by the condensation of formaldehyde with H_4-PteGlu[9]. Pteroic acid (Pte) and 4-NH_2-10-CH_3-pteroate were prepared from pteroylglutamic acid and methotrexate, respectively, by the enzymatic technique of LEVY AND GOLDMAN[10]. 10-Methyl-pteroylglutamic acid (10-CH_3-PteGlu) was prepared by anaerobic alkaline hydrolysis of methotrexate[11]. The di- and tetrahydro- derivatives of 10-methyl-pteroylglutamate (10-CH_3-H_2-PteGlu and 10-CH_3-H_4-PteGlu) were prepared as described above. 5-Methyl-5,6-dihydropteroylglutamate (5-CH_3-H_2-PteGlu) was prepared from 5-methyl-tetrahydropteroylglutamate by enzymatic peroxidation[12]. Formylation[13] of pteroylglutamic acid and dihydropteroylglutamic acid resulted in 10-formyl-pteroylglutamic acid (10-CHO-PteGlu) and 10-formyl-dihydropteroylglutamic acid (10-CHO-H_2-PteGlu), respectively.

Identification of prepared compounds was made by spectrophotometry using a Unicam SP-800 recording spectrophotometer. In those cases where the presence of antioxidants such as ascorbate or MET interfered with spectra of labile reduced compounds (*e.g.* 10-CHO-H_4-PteGlu) anaerobic Thunberg type UV cells (Hellma Ltd.) were used.

Radiochemicals and their measurement

Several types of tritiated PteGlu were employed: generally labelled G(T) obtained by catalytic exchange (1.5 Ci/mmole) or by T_2 gas exposure (5.5 mCi/mmole), as well as 3′5′9(n) T (250 mCi/mmole, 33 Ci/mmole).

Sodium boro[^{3}H]hydride (4.2 Ci/mmole), 5-[^{14}C]CH_3-H_4-PteGlu barium salt (61 mCi/mmole), 2-[^{14}C]PteGlu potassium salt (55.3 mCi/mmole), and DL-[1-^{14}C]glutamic acid (23.5 mCi/mmole) as well as the tritiated pteroylglutamates above were all purchased from the Radiochemical Centre, Amersham, Great Britain.

Tritiated H_4-[6,7-$^3H_2$]PteGlu was prepared from PteGlu and ^{3}H-labelled $NaBH_4$ according to the method of SCRIMGEOUR AND VITOLS[6]. The crude product was purified by DEAE-cellulose (Whatman DE-52) ion-exchange chromatography using a stepwise concentration gradient of 0.13–0.4 M ammonium acetate containing 0.1 % ammonium ascorbate as antioxidant. The fractions containing *d,l*-H_4-[6,7-$^3H_2$]PteGlu were pooled and reduced in volume by lyophilization. This material proved to be homogenous by TLC (R_F 0.63) and its specific activity based upon the *Lactobacillus casei* (NCIB 10463) assay with PteGlu as reference standard was *ca.* 2.9 mCi/mg.

A portion of the crude *d,l*-H_4[6,7-$^3H_2$]PteGlu was used to prepare the *d,l*-5-CHO H_4-[6,7-$^3H_2$]PteGlu. The aliquot was lyophilized to dryness, dissolved in 2.0 ml 98–100 % formic acid containing 40 μl MET and heated at 60° for 3 h[14]. The reaction mixture was reduced to 1.0 ml by lyophilization and was applied to a 1 × 20 cm column packed with a non-ionic cellulose (Whatman CF 11), which had been washed with 0.1 M formic acid containing 0.01 M MET. Elution was carried out with the same solution and the absorbance of each fraction was measured at 355 nm in a Unicam SP-500 UV-visible spectrophotometer. Fractions with absorbance values greater than 0.05 and relatively high radioactivities were pooled, lyophilized and chromatographed on a 1 × 20 cm DE-52 column as for H_4-PteGlu described above. The fractions having the greatest radioactivities were analyzed by TLC and assayed for biological activity with *L. casei* and *Streptococcus faecalis* (ATCC 8014). The major fraction was identified as *d,l*-5-CHO-H_4-[6,7-$^3H_2$]PteGlu on the basis of its activity for both *L. casei* and *S. faecalis*, its long-term stability and R_F value on TLC (0.76–0.85). The specific activity of this material was *ca.* 26 μCi/mg.

Another portion of crude H_4-[6,7-$^3H_2$]PteGlu was used to prepare 5-CH_3-H_4-[6,7-$^3H_2$]PteGlu by methods described above. A specific activity of *ca.* 650 μCi/mg was obtained.

Tritiated 7,8-H_2-[3',5',9(n)-$^3H_3$]PteGlu was prepared by reduction of [3',5',9(n)-$^3H_3$]PteGlu with sodium dithionite. Fifty microlitres (10 μCi) of tritiated PteGlu (250 mCi/mmole) were mixed with 150 μl of a 10 % (w/v) potassium ascorbate solution, pH 6.0. In this solution were dissolved 5 mg $Na_2S_2O_4$. After 5 min at 20° the mixture was cooled to 0° and the pH adjusted to 2.8 with 2 N HCl. After several minutes a precipitate of tritiated dihydropteroyl acid formed. This was sedimented, washed twice with 0.4 ml 0.005 M HCl, suspended in 0.15 ml of the same HCl solution and stored at 4° for several hours before use.

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer Model 3380. The scintillation fluid used consisted of 5.6 g 2,5-diphenyloxazole, 0.14 g 1,4-bis-4-methyl-5-phenyloxazolyl-2-benzene, 1.4 l toluene and 700 ml Triton X-100. Aqueous samples were generally measured at the ratio of 1:10 (v/v) samples: toluene–Triton X-100 scintillant. For counting proteinaceous pellets a prior incubation at 37° for 4 h with 1.0 ml Hyamine 10X Hydroxide (*p*-diisobutylcresoxyethoxyethyl-dimethylbenzylammonium hydroxide) was used. Counting efficiency for ^{3}H and ^{14}C was estimated by AES (automatic external standardization) ratio through correction curves employing *n*-[1,2-$^3H_2$]hexadecane and [*Me*-^{14}C]toluene as internal standards.

Microbiological assays and γ-glutamyl carboxypeptidase

The chloramphenicol-resistant strain of *L. casei* (NCIB 10463) was used throughout[16]. Pteroylglutamic Acid (PGA) Assay Broth (Baltimore Biological Laboratories)

was used at half the recommended strength and contained 0.1 % (w/v) ascorbic acid, 10–20 μg/ml chloramphenicol (chloromycetin of Parke Davis) and 0.01 % Tween-80. Both analytical samples and standards (0.05–0.8 ng PteGlu per tube) were added by micropipette to 5.0 ml of assay medium contained in disposable plastic tubes. Turbidity was measured after 36- to 48-h incubation at 37° in a Unicam SP-600 spectrophotometer at 530 nm.

S. faecalis (ATCC 8043) was used with Folic Acid Assay Medium (Difco) also prepared to half-strength and containing 0.1 % ascorbic acid. Samples and folate standards (0.5 to 4 ng per tube) were added to 5.0 ml volumes of medium as above, but tubes were autoclaved 116° for 15 min prior to inoculation. After incubation for 24 h at 37° turbidity was measured as for *L. casei*.

Chicken pancreas conjugase (γ-glutamyl carboxypeptidase) was prepared according to the method of BANERJEE AND CHATTERJEA[17]. Conjugase from the intestinal mucosa of guinea-pig was also prepared[18]. Conjugase activity was assayed with *S. faecalis* and synthetic pteroyltriglutamate ($PteGlu_3$)[19]. As $PteGlu_3$ does not support the growth of this microorganism in concentrations below 1.6 ng/ml, hydrolysis of one or two peptide links yields products with much greater *S. faecalis* activity, at least sixteen-fold in the case of PteGlu. *L. casei* activity was also used as an indication of conjugase activity since pteroyltetraglutamate and higher homologues ($PteGlu_{4-7}$) fail to support its growth.

Transport of labelled folates into blood cells

Blood collected from volunteers into heparinized syringes was stored at 4° or used immediately. Several different concentrations of the radioactive compounds were employed. All experiments were performed with 2 ml of blood. Samples of 0.2 ml were taken immediately after addition of the labelled folates and after various intervals of incubation at 37° and in the case of dihydropteroylglutamate under nitrogen atmosphere. Plasma was separated by centrifugation in 50 × 6 mm tubes at 4,400 × *g* for 5 min, the cells were washed twice with saline, lysed by the addition of an equal volume of sodium phosphate–ascorbate buffer, pH 7.0, followed by freezing and thawing. This mixture was centrifuged at 4,400 × *g* for 30 min to sediment a pellet and the supernatant ("lysed blood") was removed. The plasma was treated with an equal volume of cold 20 % (w/v) trichloroacetic acid, centrifuged and the supernatant removed. Small samples (1–50 μl) of the deproteinated plasma, washed and lysed blood were added to 10 ml scintillation fluid and counted. The pellets of plasma proteins and blood were dissolved by incubation with 1.0 ml Hyamine 10X Hydroxide at 37° prior to counting.

Metabolism of labelled folates in plasma and blood cells

Lysed blood samples obtained at the various intervals as well as deproteinated plasma were analyzed by TLC, the former after treatment with conjugase preparations to convert any subsequent polyglutamate metabolites to the pteroylmonoglutamate level. Samples were generally applied in volumes of < 12 μl/cm for layers 0.5 mm thick, and developed in 3 % NH_4Cl–0.5 % MET. One-centimetre bands were scraped off the plates and filtered with 1.0 ml of 10 % ammonium hydroxide through Whatman GFA filters. The relative efficiency of recovery of labelled materials from the thin-layer plates with this procedure was compared with direct addition of cellulose to the scintillation fluid.

RESULTS AND DISCUSSION

In our hands the non-ionic cellulose powder (MN 300 UV_{254}) with fluorescent indicator proved to be the most effective support for separating the pteroylmonoglutamates as well as several related compounds (Fig. 1). The R_F values of these compounds, chromatographed in the more effective solvent systems employed, are shown in Table I–III. Very few of the several solvents tested exhibited any special merit. For example, a good resolution of PABGlu and Glu was obtained with the system butanol–water–ethanol–acetic acid and the system citrate–ammonium hydroxide saturated with isoamyl alcohol gave well compacted spots with the pterins.

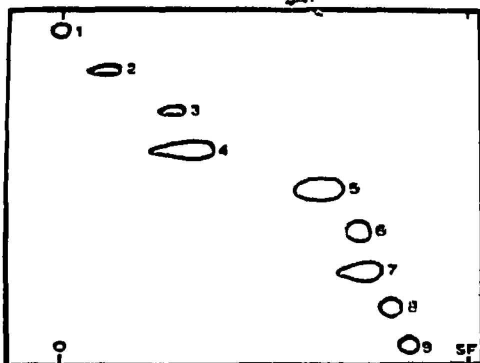


Fig. 1. Schematic representation of thin-layer chromatographic separation of pteroylmonoglutamates and pteroate on MN 300 UV_{254}. 1 = Pte; 2 = H_2-PteGlu; 3 = PteGlu; 4 = 5,10-CH = H_4-PteGlu; 5 = H_4-PteGlu; 6 = 5-CHO- or 5-HCNH-H_4-PteGlu; 7 = 5,10-CH_2-H_4-PteGlu; 8 = 5-CH_3-H_4-PteGlu; 9 = 10-CHO-H_4-PteGlu.

The most important feature of the ammonium chloride system (Table I) is that in addition to resolving PteGlu, H_2-PteGlu, and H_4-PteGlu, which had been previously achieved by an ion-exchange TLC system[4], 5- and 10-CHO-H_4-PteGlu and 5,10-CH = H_4-PteGlu are also resolved with mean R_F values of 0.73, 0.82, and 0.32, respectively. The 5-CH_3-H_4-PteGlu and 10-CHO-H_4-PteGlu derivatives are not adequately separated but can be distinguished by a differential microbiological assay (*viz. L. casei* and *S. faecalis*) or by conversion of the latter compound to 5,10-CH=H_4-PteGlu by acidification. Similarly, while the 5-CHO- and 5-HCNH-H_4-PteGlu exhibit nearly identical R_F values by acidification, the former compound may be converted to the 5,10-CH=H_4-PteGlu and thus separated.

Although Pte and H_2-PteGlu are completely resolved, the tailing portion of 5,10-CH=H_4PteGlu is not completely separated from PteGlu. While PABA and PABGlu are well separated, PABGlu and Glu are not. With the exception of L-biopterin and L-neopterin, which possess 3- and 4-hydroxyl functions, pterins and other pteridines tail excessively in the NH_4Cl system.

The separation of naturally occuring pteroylmonoglutamates obtained with the NH_4Cl system can be improved by rechromatography of 20 × 20 cm plates in a second dimension with one of the 0.1 *M* phosphate buffers (Table I). If improvement of overall resolution is desired pH 6.0 is recommended, whereas separation of PteGlu, H_2-PteGlu and 5, 10-CH = H_4-PteGlu is increased by use of pH 7.0. However, it

TABLE I

TLC OF NATURALLY OCCURRING FOLATES (PTEROYLMONOGLUTAMATES) AND PTEROATE ON MN 300 UV_{254} POWDER

Compound[a]	NH_4Cl[b]	Sodium phosphate[c]		Detection[d]
		A	B	
Pte	0[f]	0	0	Q/A
H_2-PteGlu	10	26	24	BF/BF
PteGlu[e]	24	56	40	Q/A
5,10-CH = H_4-PteGlu	32[g]	79[g]	45[g]	Q-BF/BF
H_4-PteGlu[e]	56	70	59	BF/BF
5-CHO-H_4-PteGlu[e]	72	85	76	Q/YBF
5-HCNH-H_4-PteGlu	72	85		Q/
5,10-CH_2-H_4-PteGlu	75[g]		82[g]	Q/
5-CH_3-H_4-PteGlu[e]	80	86	79	Q/
10-CHO-H_4PteGlu	82	81	82	Q/
5-CH_3-H_2-PteGlu	87	58	84	Q/
10-CHO-PteGlu	70	58		Q/
10-CHO-H_2-PteGlu	73	72		Q/BF

[a] All tetrahydro compounds and 5-CH_3-5,6-H_2-PteGlu are racemic mixtures with the exception of *l*-5-formimino-tetrahydropteroylglutamate.

[b] 3.0% (w/v) NH_4Cl containing 0.5% (v/v) 2-mercaptoethanol as antioxidant (pH 6.2).

[c] (A) 0.1 *M* sodium phosphate buffer pH 7.0, (B) 0.1 *M* sodium phosphate buffer pH 6.0 both containing 0.5% (v/v) 2-mercaptoethanol.

[d] Exposure to UV light 254 nm/366 nm; abbreviations: A = absorption, B = blue, F = fluorescence, Q = quench and Y = yellow.

[e] Compounds also applied in their radioactive forms.

[f] Mean R_F values × 100 of two to eleven determinations.

[g] Elongated spot, *i.e.* greater than 20 mm.

TABLE II

TLC OF PTEROYLMONOGLUTAMATES AND RELATED COMPOUNDS ON MN 300 UV_{254} POWDER

Compound[a]	NH_4Cl[b]	Sodium phosphate[c]		Detection[d]
		A	B	
4-NH_2-10-CH_3-Pte	10[f]	31		Q/A
4-NH_2-10-CH_3PteGlu[e]	67	67	74	Q/A
10-CH_3PteGlu	62	84	80	Q/A
10-CH_3-H_2-PteGlu	38			BF/BF
10-CH_3-H_4-PteGlu	70			Q/YBF
PABA	56	85	73	Q/
Glu	93	98	94	Ninhydrin positive
PABGlu	91	94	97	Q/
BA	79	93	84	Q/

[a] Abbreviations: PABA = *p*-aminobenzoic acid; PABGlu = *p*-aminobenzoylglutamate; BA = benzoic acid.

[b,c,d,e] see Table I.

[f] Mean R_F values × 100 of two to five determinations.

TABLE III

TLC OF SEVERAL PTERINS AND PTERIDINES ON MN 300 UV_{254} POWDER

Compounds[a]	NH_4Cl[b]	*Na phosphate*[c]	*Cit*:NH_3[d]	*Detection*[e]
Pt-6-COOH	28[f,g]	56	42	BF/BF
Pt-7-COOH	28[g]	49[g]	49	BF/BF
Pt-6-OH	50	46[g]	43	Q
Pt-6-OH-7-CH_3	33[g]	26[g]	43	BF/YBF
L-Neopterin	59	55[g]	68	BF/BF
L-Biopterin	64	63[g]	66	BF/BF
Pt-7-OH	33	36		
4-OH-pteridine	55[g]	62[g]		
2,4-OH-pteridine	64	81		
2-NH_2-4-OH-pteridine (Pt)	26	03	37[g]	BF/BF
2-SH-pteridine	65	74		

[a] Pt = 2-NH_2-4-OH-pteridine.
[b] See Table I.
[c] 0.1 *M* sodium phosphate buffer pH 7.0 containing 0.5% (v/v) 2-mercaptoethanol.
[d] 5.0% citrate adjusted to pH 9.0 with ammonium hydroxide, saturated with isoamyl alcohol.
[e] See Table Id.
[f] R_F values × 100.
[g] Elongated spot 20 mm.

should be borne in mind that many of the natural derivatives investigated, particularly H_2-PteGlu, H_4-PteGlu, and 5,10-CH_2-H_4-PteGlu, are highly susceptible to oxidation by molecular oxygen, thus plates cannot be air-dried prior to chromatography in the second dimension with any assurance of their continued degree of purity. This, we feel, may be a major practical obstacle to any routine two-dimensional chromatographic separation of these labile derivatives. Also, without tritium plate scanning equipment analysis of tritiated metabolites in the manner described (*i.e.*, by liquid scintillation counting) would be considerably lengthened in a two-dimensional system. This might severely limit the use of the technique in kinetic studies.

The cation-exchange layers recommended by COPENHAVER AND O'BRIEN[4] were found less effective than MN 300 UV_{254} powder alone with NH_4Cl and the various phosphate solvent systems employed above. Somewhat more promising in certain respects were the mixtures of the Bio-Rad TLC ion-exchange cellulose (D & CM) and MN 300 UV_{254} powder, but again none of these systems (developed with NH_4Cl and phosphate buffers) compared favourably with the overall resolution effected by NH_4Cl and NM 300 UV_{254} alone. The presence of a fluorescent indicator in the powder obviates the need for spray reagents in the detection of pteroylmonoglutamates and related compounds with the exception of glutamic acid. It was found that while 2-mercaptoethanol absorbs light at 254 nm, the use of 0.5 % (v/w) in the chromatographic solvents did not interfere with the fluorescent indicator. This cannot be said of the antioxidant L-ascorbate, which has a much higher extinction and quenches plate fluorescence excessively at 0.1 % (w/v). The limit of detection by quenching at 254 nm is generally less than 3.0 μg after development. Compounds which exhibit fluorescence when irradiated at 360 nm can often be detected in submicrogram quantities. Development time is usually 80–90 min at 20°.

Although the sensitivity of UV detection is relatively good by chemical stan-

dards it is far too insufficient to detect pteroylmonoglutamate derivatives directly in biological materials unless coupled to bioassay or bioautographic techniques. This does not detract from the genuine utility of the technique in assessing the chemical purity of folate derivatives, especially where their synthesis is involved.

Alternatively, radioactive folates usually have specific activities high enough to allow detection of 10 ng or less and the TLC techniques lend themselves more easily to biological and biomedical experimentation with labelled folates, some of which are available commercially and others which may be easily synthesized by standard techniques.

Radioactive materials were most conveniently recovered from TLC plates simply by scraping bands of cellulose layers into scintillation vials followed by one volume of 10 % ammonium hydroxide and ten volumes of scintillation fluid. Recovery of tritiated folate by this technique was about 45–55 % and [^{14}C]folate in excess of 70 % of that applied to the plate.

Investigation of naturally occurring folate derivatives was pioneered by NORONHA AND SILVERMAN[20] with the development of column chromatographic techniques capable of separating numerous derivatives. The identification of the various folate derivatives in plasma, red cells or whole blood has been the subject of several studies[21–29]. It has been demonstrated that *L. casei* shows a much greater response to the folate derivatives of serum than does *S. faecalis* and that this differential response is due to presence of 5-CH_3-H_4-PteGlu. Polyglutamyl derivatives do not appear to be present to any significant extent in serum[27]. Relatively large amounts of folates were found in red cells by TOENNIES, USDIN, and co-workers[23–26,29], who showed the presence of reduced derivatives active for *L. casei* but not for *S. faecalis*. These are 5-CH_3H_4-PteGlu and its di- and triglutamyl homologues. The activity for *L. casei* was observed to increase after incubation of haemolysates with plasma, or upon autolysis. This has been shown to be the result of plasma enzymes, γ-glutamyl carboxypeptidases (conjugases), acting on red cell pteroylpolyglutamates.

In vivo experiments on folate uptake have been concerned with measuring either urinary excretion or increases in serum folate. Tritiated folate, injected intravenously, has been shown to be very rapidly cleared from the plasma and as it is not accounted for by urinary excretion it is assumed to be taken into the intracellular space[30]. Comparatively little is known about the uptake of various folate derivatives by erythrocytes. HERBERT AND ZALUSKY[3] found that after intravenous injection of folate there was no significant increase in the folate content of erythrocytes estimated by *L. casei* and *S. faecalis* activities. They observed that a sample of erythrocytes containing 26 % reticulocytes contained nine times more folate derivatives active for *L. casei* than another sample containing 6 % of reticulocytes. They concluded that the mature erythrocyte is relatively impermeable to folic acid.

NEAL AND WILLIAMS[3] showed that 24 h after the injection of tritiated folic acid to rats less than 1 % of the injected radioactivity was found in the erythrocytes. There have been no reports on the uptake and fate of reduced folate derivatives by erythrocytes although 5-CH_3-H_4-PteGlu has been shown to be actively transported into leucocytes[31].

IZAK *et al*[1]. found that 1–2 % of 2–10 pg of [^{3}H]PteGlu was taken up *in vitro* by human erythrocytes containing up to 50 % reticulocytes. CORCINO *et al*[32], using incubation periods up to 4 h, showed that the temperature-dependent uptake of [^{3}H]Pte-

Glu by human bone marrow cells *in vitro* was approximately six-fold greater than that of reticulocytes and uptake of 5-CH_3-H_4-[^3H]PteGlu appeared to be twice that observed with [^3H]PteGlu. These authors suggest two mechanisms for folate uptake by human reticulocytes and bone marrow cells, *viz.* an energy-dependent active carrier mechanism and a passive diffusion-like mechanism.

Incorporation of 5-[^{14}C]CH_3-H_4-PteGlu and 5-CH_3H_4-[6,7-$^3H_2$]PteGlu into blood cells

Separate experiments using different concentrations of 5-[^{14}C]CH_3-H_4-PteGlu and 5-CH_3H_4[6,7-$^3H_2$]PteGlu showed similar results. There was a steady loss of radioactivity from the plasma and a corresponding increase in the lysed blood fraction with increasing incubation times. After 12 h as much as 9 % of the radioactivity initially in the plasma was incorporated into the blood cells (Table IV). TLC fractionation of both plasma and lysed blood each resulted in single peaks with R_F values identical with that for 5-CH_3H_4-PteGlu. Incubation of the lysed blood with a conjugase preparation active for PteGlu did not affect the R_F value.

TABLE IV

DISTRIBUTION OF RADIOACTIVITY IN BLOOD AFTER INCUBATION WITH LABELLED 5-METHYL-TETRAHYDROFOLATE

Compound conc. in whole blood	*5-CH_3-H_4-[6,7-$^3H_2$]PteGlu*		*5-[^{14}C]CH_3-H_4-PteGlu*
	Normal 1.95 μg/ml	*Polycythaemic, 1.95 μg/ml*	*Normal, 11 μg/ml*
Plasma	676,480[a]	660,180[a]	582,000
Saline Wash 1	117,540	94,620	192,000
Saline Wash 2	38,220	27,900	12,300
Saline Wash 3	19,560		
Lysed blood	63,140	64,938	80,000
%	6.9	7.4	9.1
Time, h	10.5	11	12

[a] d.p.m.

Incorporation of [^3H,^{14}C]PteGlu

Several experiments employing a wide range of folate concentrations showed that this compound is transported to varying degrees into normal mature blood cells (Table V). The highest degree of radioactivity incorporation was found to be 6.23 % in 24 h, whereas the lowest degree of incorporation observed was slightly less than 2 % in 12 h. While IZAK *et al*[1]. observed incorporation of less than 1 % in 1 h with mature red cells (0.1–0.5 % reticulocytes) and greater than 2 % in 30 min with reticulocyte-rich (50–60 %) blood, they were unable to determine whether radioactivity recovered from the incubated cells represented pteroylglutamate or some product of its metabolism during or subsequent to incorporation.

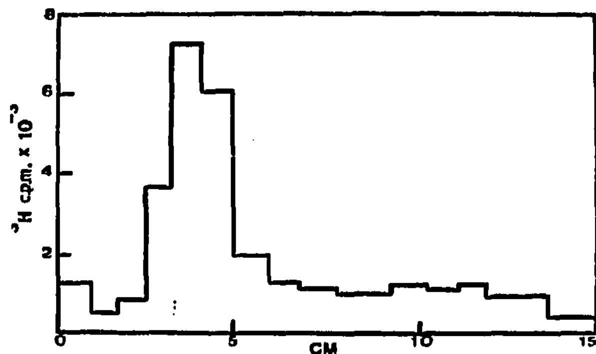
The results of our TLC analyses of lysed blood fractions obtained subsequent to incorporation of either PteGlu or 5-CH_3-H_4-PteGlu (Figs. 2 and 3), indicate that little or no transformation of these metabolites occurs up to 12 h. That is, the R_F values of the single radioactive components in the lysed blood fractions were identical to those of PteGlu and 5-CH_3-H_4-PteGlu, respectively, both before and after treatment with

TABLE V

DISTRIBUTION OF RADIOACTIVITY IN BLOOD AFTER INCUBATION WITH LABELLED PTEROYLMONOGLUTAMATE (FOLATE)

Compound, conc. in whole blood	*[3',5',9-$^3H_3$]PteGlu*		*[2-^{14}C]PteGlu normal, 1.2 μg/ml*
	Normal, 85 ng/ml	*Polycythaemic, 93 ng/ml*	
Plasma	559,515[a]	2,546,700[a]	91,000
Saline Wash 1	344,220	202,560	39,300
Saline Wash 2	68,280	27,480	3,060
Saline Wash 3	51,420		1,410
Lysed blood	33,462	92,189	3,360
%	3.36%	3.21	2.4
Time, h	10	11	12

[a] d.p.m.



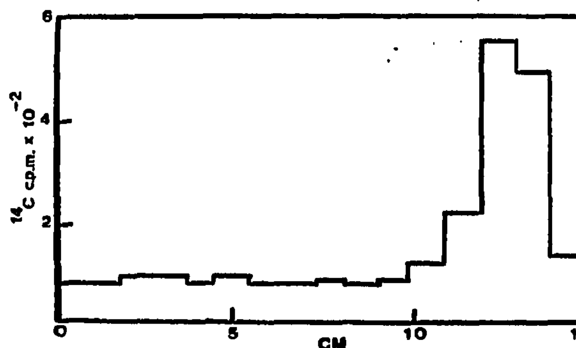


Fig. 2. Thin-layer chromatography of lysed blood after 24-h incubation of whole blood with [3',5',9-$^3H_3$]PteGlu.

Fig. 3. Thin-layer chromatography of lysed blood after 12-h incubation of whole blood with 5-[^{14}C]CH_3-H_4-PteGlu.

an active conjugase preparation, which yields pteroylmonoglutamates from pteroylpolyglutamates. In the usual solvent systems used, the R_F values of pteroylglutamate and probably its derivatives, increase progressively as glutamyl residues are added (*e.g.* R_F 0.93 for $PteGlu_7$ in 3% NH_4Cl–0.5% MET). Therefore, one might expect higher glutamyl homologs of PteGlu and 5-CH_3-H_4-PteGlu to exhibit higher R_F values, and to shift to lower values after conjugase treatment. Furthermore, TLC analyses of the deproteinated plasma fractions indicate that these metabolites were also untransformed in the plasma throughout the course of observation.

Incorporation of H_2-PteGlu, H_4-PteGlu and 5-CHO-H_4-PteGlu into blood cells

The results obtained to date with H_2-[3',5',9-$^3H_3$]PteGlu and H_4-[6,7-$^3H_2$]PteGlu have been inconclusive probably because of their marked instability *in vitro*, even under nitrogen atmosphere. The radioactivity of dihydropteroylglutamate in the plasma did not decrease significantly and radioactivity above background level could

not be detected in the lysed cell fraction. On the other hand, radioactivity due to [6,7-$^3H_2$]tetrahydrofolate or its decomposition products was incorporated into the lysed blood fraction to the extent of 9.8 % after 11-h incubation. The concentration of ^3H activity in the lysed blood was insufficient to allow TLC analysis. The plasma, however, after 8-h incubation exhibited approximately equal ^3H activity at R_F 0.8 and R_F 0.6. Since the oxidation of H_4-PteGlu usually leads to a mixture of pteridines of low R_F value (*e.g.* Pt-6-COOH, Pt-6-CHO) and *p*-aminobenzoylglutamate of high R_F value), these two fractions are not considered to be degradation products. The latter material is probably H_4-PteGlu (mean R_F 0.56), the former could be either 5-CHO-H_4-PteGlu or 5-CH_3-H_4-PteGlu; whether this reflects metabolic conversion by the blood cells or extracellular action is still uncertain.

Although many of the concentrations of folate compounds employed in this study are not unphysiological in the usual sense they are certainly far above the concentrations of these metabolites that might normally be found in the blood even after food. In order to use physiological concentrations effectively with subsequent chromatographic fractionation of lysed blood, high specific activities approaching 10 μCi/μg are required. As specific activities this high are only commercially available in [3',5',9-$^3H_3$]PteGlu produced by catalytic dehydrohalogenation with tritium gas, future synthesis of reduced derivatives for transport and metabolic studies should either rely on this as a starting material or attempts should be made to prepare reduced folates by PtO_2-catalyzed hydrogenation with tritium gas.

The incorporation of labelled folate derivatives as described above does not reveal the degree of net transport of these compounds into the blood cells but rather sets an upper limit. As in the case of normal blood the uptake of radioactivity seldom exceeded 9 % of that available in the plasma, it was not possible to show unequivocally a net uptake of a given compound based upon a decrease in plasma *L. casei* activity during the incubation period. Also it is unknown whether in the case of racemic mixtures (*i.e.* (±)-5-CH_3-H_4-PteGlu and (±)-H_4-PteGlu) the biologically active diastereomer is preferentially incorporated.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Biomedical Research Trust. We wish to thank Miss C. HOULIHAN, who prepared the pteroic acid, pteroyltriglutamate and pteroylheptaglutamate.

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