The Identification of the Folate Conjugates Found in Rat Liver 48 h after the Administration of Radioactively Labelled Folate Tracers

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About 70% of the radioactivity retained in the livers of rats dosed 48 h earlier with radioactively labelled folate was incorporated into two folate conjugates. The major derivative was purified and isolated by Sephadex G-15, DEAE-cellulose and DEAE-Sephadex ion-exchange column chromatography and paper chromatography. It was identified as 10-formylpteroylpentaglutamate by a combination of spectral, microbiological, chemical and chromatographic techniques. The minor conjugate, though less well characterized, exhibited similar properties and was assigned the structure 10-formylpteroyltetraglutamate. 10-Formylpteroylpentaglutamate (2.0 nmol/g) and 10-formylpteroyltetraglutamate (0.25 nmol/g) comprised about 20% of the total endogenous hepatic folate as determined by microbiological assay (*Lactobacillus casei* after conjugase treatment).

Mammalian tissues contain substantial amounts of conjugated folate derivatives (Blakley, 1969). These conjugates are not available to the micro-organisms commonly employed in the assay of folate derivatives unless subjected to prior treatment with preparations containing the hydrolytic folate 'conjugase' enzymes (glutamate carboxypeptidase, EC 3.4.12.10), and are thus described as poly- γ -glutamate conjugates (Herbert, 1963; Bird *et al.*, 1965). Kinetic studies *in vitro* with synthetic pteroyl- γ -glutamate derivatives have indicated that folate conjugates may function as coenzyme forms *in vivo* (Hoffbrand, 1976). However, direct evidence of either the specific identity or function of the folate conjugates found *in vivo* has been hampered by the lack of definitive methods of analysis of tissue folates.

Early studies utilized ion-exchange chromatography combined with microbiological assay and radioactively labelled tracers to separate out the conjugated and free folate of tissue extracts (Noronha & Silverman, 1962; Bird *et al.*, 1965). However, resolution of synthetic folate monoglutamates and folate

polyglutamates is poor, the state of reduction and of C₁ substitution being more important than glutamate chain length in determining elution position (Kisliuk *et al.*, 1974; Barford *et al.*, 1977). The use of Sephadex gel filtration, though allowing the separation of folate monoglutamates from higher-molecular-weight forms (Shin *et al.*, 1972), suffers similar drawbacks, since folate derivatives undergo non-specific interactions with the gel and thus are not eluted simply according to molecular weight (Baugh *et al.*, 1974; d'Urso-Scott *et al.*, 1974). Both these chromatographic techniques rely on the use of co-chromatography with authentic standards before and after treatment with often grossly impure conjugase preparations, e.g. human serum (Reed *et al.*, 1977); only recently has a folate conjugase been isolated in a pure form (Rao & Noronha, 1977).

To overcome the problems of anomalous chromatographic behaviour conferred by the pteridine moiety of the folate molecule, methods have been described in which tissue folates are extracted and chemically cleaved at the C-9–N-10 bond. Either Zn/HCl reduction (Leslie & Baugh, 1974) or KMnO₄ oxidation (Houlihan & Scott, 1972) is employed to yield *p*-aminobenzoyl-L-glutamate derivatives, which can then be separated by ion-exchange chromatography to establish the conjugate chain length. However, both methods fail to provide evidence of the nature of the pteridine moiety, and, although pteroylglutamic acid readily undergoes such cleavage reactions, the formyl and methyl deri-

Abbreviations used: PteGlu, pteroylglutamate; PteGlu₂, pteroyldiglutamate; PteGlu₃, pteroylheptaglutamate; 10-HCO-PteGlu, 10-formylpteroylglutamate; 10-HCO-PteGlu₂, 10-formylpteroyldiglutamate; 10-HCO-PteGlu₄, 10-formylpteroyltetraglutamate; 10-HCO-PteGlu₅, 10-formylpteroylpentaglutamate; 10-HCO-H₄PteGlu, 10-formyl-5,6,7,8-tetrahydropteroylglutamate; 5-CH₃-H₄PteGlu, 5-methyl-5,6,7,8-tetrahydropteroylglutamate.

vatives of tetrahydrofolate, the naturally occurring coenzyme forms, fail to do so under the conditions described (Lewis & Rowe, 1977; Maruyama *et al.*, 1978; Baugh *et al.*, 1979).

In our laboratory we have attempted to develop non-destructive unambiguous methods of analysis to identify mammalian folates (Connor *et al.*, 1977). Previous investigations have shown that, after the administration of radioactively labelled PteGlu to rats, substantial amounts of the retained radioactivity of the liver behave as a high-molecular-weight derivative, i.e. a folate conjugate, on gel filtration (Barford *et al.*, 1977). The present paper is a detailed study of the isolation and characterization of the folate conjugates of rat liver found 48 h after the administration of folate tracers to label the folate pool *in vivo*.

Materials and Methods

Chemicals and reagents

The folates 10-HCO-PteGlu, 10-HCO-H₄ PteGlu, 5-CH₃-H₄PteGlu, PteGlu, [3',5',9(n)-³H]PteGlu (specific radioactivity 500 Ci/mol) and [2-¹⁴C]-PteGlu (specific radioactivity 58.2 Ci/mol) were synthesized or obtained commercially as described by Barford *et al.* (1977). The commercially obtained [3',5',9(n)-³H]PteGlu has recently been shown to contain about 20% of the tritium at the C-7 position by tritium n.m.r. and is now supplied as [3',5',7,9-³H]PteGlu (The Radiochemical Centre, Amersham, Bucks., U.K.). Authentic [G-³H]PteGlu, synthesized by the method of Godwin *et al.* (1972) was a gift from Professor I. H. Rosenberg (Department of Gastroenterology, University of Chicago, Chicago, IL, U.S.A.). Chicken pancreas folate conjugase was purified by the method of Laskowski *et al.* (1945). A crude preparation of rat intestinal folate conjugase was obtained by homogenizing rat small intestine (prewashed in ice-cold 0.15 M-NaCl) in 4 vol. of 0.1 M-sodium phosphate buffer, pH 6, containing sodium ascorbate (2%, w/v) (after the method of Godwin *et al.*, 1972). The homogenate was centrifuged at 3000 g in a bench centrifuge and the supernatant was harvested and stored frozen in 1 ml portions at -15°C. The extract retained folate conjugase activity under these conditions for at least 6 months. All other general laboratory reagents used were of AnalaR grade or its equivalent.

Column chromatography

DEAE-cellulose ion-exchange chromatography (column dimensions 2 cm × 50 cm) with elution with a linear 0–1.2 M-NaCl gradient in 0.05 M-sodium phosphate buffer, pH 7, and Sephadex G-15 gel filtration (column dimensions 2 cm × 50 cm) were performed as described by Barford *et al.* (1977). DEAE-Sephadex ion-exchange chromatography was

performed by packing glass columns (2 cm × 50 cm), plugged with glass-wool, with a slurry of pre-equilibrated DEAE-Sephadex A-25 (Pharmacia AB, Uppsala, Sweden) in 0.05 M-sodium phosphate buffer, pH 7, containing dithiothreitol (5 mg/100 ml). After application of samples (5–50 ml), columns were eluted with a linear 0–1.5 M-NaCl gradient in the equilibration buffer. T.l.c. was performed with 20 cm × 20 cm Polygram cellulose plates (Camlab, Cambridge, U.K.) developed in the dark by the ascending technique.

Isolation of liver folate conjugate

Two separate experiments were performed. In an initial study (extraction A) the radioactively labelled folate conjugate was recovered from the livers of 60 rats (200–250 g) two of which had been dosed orally 48 h earlier with [2-¹⁴C]PteGlu (100 µg/kg body wt.) to enable a direct comparison with [³H]PteGlu. A second more detailed extraction (extraction B) was performed with the livers of 100 rats (150–200 g), some dosed 48 h earlier with [2-¹⁴C]PteGlu (2 µCi/rat, 24 rats dosed) or [3',5',9(n)-³H]PteGlu (5 µCi/rat, 13 rats dosed) to provide a dual-labelled radiotracer, to isolate a larger quantity of the folate conjugates for more detailed structural studies.

(a) *Preparation of crude extract from liver.* Liver was extracted by the following procedure (values in parentheses refer to extraction B). Rats were killed by cervical dislocation, and the livers (total weight 800 g containing 6.43 µCi of ¹⁴C and 13.65 µCi of ³H of the administered radioactivity) were rapidly excised, washed in ice-cold 0.15 M-NaCl, chopped and dropped into 3 vol. of boiling 0.05 M-sodium phosphate buffer, pH 7, to rapidly inactivate endogenous folate conjugase, containing sodium ascorbate (2%, w/v) and dithiothreitol (5 mg/100 ml) to decrease degradation of labile folates during extraction (Bird *et al.*, 1965; Shin *et al.*, 1972). After 10 min at this temperature (95–100°C) the extract was rapidly cooled and homogenized at 4°C in a Moulinex blender. The homogenate was centrifuged for 5 min in a bench centrifuge (3000 g) and the supernatant recovered. The pellet was washed by rehomogenization in the extraction buffer, and the washings and original supernatant were combined. Residual soluble protein was removed by the addition of solid trichloroacetic acid (10 g/100 ml). The mixture was centrifuged, and the supernatant was recovered and adjusted from pH 4.5 to pH 7 with conc. NaOH. The neutral supernatant (1900 ml) was freeze-dried, to decrease the volume, and the residue was redissolved in 150 ml of 0.01 M-sodium phosphate buffer, pH 7, at 4°C. The mixture was filtered on Whatman no. 1 chromatography paper to remove gelatinous inorganic salts (this debris contained no measurable radioactivity), and the filtrate (extract) was retained for chromatographic

purification. Measurement of the radioactivity of the extract showed that 85% of the liver radioactivity could be extracted by this technique.

(b) *Purification of the crude extract by chromatography.* Portions (20 ml) of extract were applied to Sephadex G-15 columns (2 cm × 60 cm) to separate out low-molecular-weight radioactive derivatives and unlabelled impurities and to desalt. Fractions containing radiolabelled high-molecular-weight folate conjugate, i.e. those eluted near to the void volume, were collected and pooled. The pooled eluates were then sequentially chromatographed on both DEAE-cellulose and DEAE-Sephadex anion-exchange columns (2 cm × 50 cm) to remove the bulk of the remaining u.v.-absorbing impurities. Ion-exchange resolved the folate conjugate into a major (I) and a minor (II) component. These were only partially resolved on DEAE-cellulose (being eluted at 0.54 M-NaCl and 0.6 M-NaCl respectively), but separation was almost complete on DEAE-Sephadex (being eluted at 0.6 M-NaCl and 0.7 M-NaCl respectively). Fractions containing each component were pooled and desalted on a Sephadex G-15 column (2 cm × 60 cm) eluted with distilled water, metabolites I and II being eluted at K_{av} 0.18 and K_{av} 0.24 respectively. On being freeze-dried the eluates yielded 200–600 mg of a white powder, shown to be largely inorganic phosphate salts on elemental analysis, presumably derived from the buffers used.

Folate conjugate could be isolated as a homogeneous material by further purification by paper chromatography (Whatman no. 3) with propanol/acetic acid/water (200:1:99, by vol.) as the solvent, developed by the descending method. This system removed the remaining u.v.-absorbing impurity (λ_{max} 263 nm at pH 7) (Friedkin *et al.*, 1975). At this stage the major derivative (I) could be detected as a blue-fluorescent band (R_F 0.14) visible under u.v. light. Throughout the purification recoveries were followed by using both the radioactivities and the activities in the microbiological assay (with *Lactobacillus casei* before and after folate conjugase treatment) of the extracts.

Electrophoresis

Electrophoresis, used for product analysis and to demonstrate homogeneity, was performed with a Shandon Southern flat-bed tank (Shandon Southern Products, Runcorn, U.K.) with 2.5 cm × 16 cm paper strips (Whatman no. 1). Trial experiments demonstrated that adequate separation of folate monoglutamate markers with minimal chemical decomposition could be achieved by using 0.05 M-sodium phosphate buffer, pH 7, containing dithiothreitol (5 mg/100 ml), as the electrolyte buffer, at constant voltage (20 V/cm) for 60 min.

Enzymic degradation studies

Authentic [^3H]PteGlu, and the major product of extraction A (labelled with ^{14}C) were incubated with chicken pancreas folate conjugase (0.1 ml) or with the rat intestinal folate conjugase preparation (1 ml). All incubations were performed in 0.1 M-sodium phosphate buffer, pH 7, containing sodium ascorbate (2%, w/v) in conical flasks loosely stoppered with cottonwool plugs, and the reactions were allowed to proceed for 2 h at 37°C in a total volume of 10 ml. The liver extract and PteGlu, were incubated both together and separately with folate conjugase to check on the presence of natural folate conjugase inhibitors, as have been reported in yeast extracts (Rosenberg & Godwin, 1971), that may have co-purified with the liver extract. A control in which the substrates were incubated in the absence of folate conjugase was performed to ascertain any spontaneous chemical degradation. The products of each incubation were then examined by column chromatography on Sephadex G-15 and DEAE-cellulose as described above and differential microbiological assay with *L. casei*, *Streptococcus faecalis* and *Pediococcus cerevisiae*.

In a second study the major component isolated from extraction B (BI) was incubated with the rat intestinal folate conjugase preparation for a more rigorous product determination. Component BI (35 nmol) was incubated with rat intestinal folate conjugase (1 ml) as described above for 1 h. The reaction mixture was then concentrated by freeze-drying, and the product was characterized by Sephadex G-15 gel filtration, DEAE-cellulose ion-exchange, t.l.c. in three solvents [1, propan-1-ol/aq. NH_3 (sp.gr. 0.880)/water (200:1:99, by vol.); 2, aq. 3% (w/v) NH_4Cl ; 3, 0.05 M-sodium phosphate buffer, pH 7], thin-layer gel filtration and electrophoresis as described above. The product was compared in each case with internal authentic 10-HCO-PteGlu and other folate monoglutamate markers.

Other methods

Differential microbiological assay and bioautography with *L. casei*, *S. faecalis* and *P. cerevisiae* (Herbert, 1966) and thin-layer gel filtration with Sephadex G-15 as the support medium and a Dextran Blue reference were performed by Dr. R. J. Leeming (General Hospital, Birmingham, U.K.). Amino acid compositions were determined with a Locarte automatic analyser having a minimum acceptable sensitivity of 1 nmol of amino acid (Dr. J. E. Fox, Macromolecular Analysis Service, University of Birmingham, Birmingham, U.K.), with sample sizes greater than 10 times the minimum acceptable value. Radioactivity was determined as described by Connor *et al.* (1979). Fluorescence spectra were determined with an Aminco-Bowman

spectrofluorimeter and u.v. absorbance with a Pye Unicam SP. 1750 recording spectrometer.

Results

Recoveries during purification and the hepatic concentration of the folate conjugates

The recovery of the folate conjugates during purification (extraction B) is summarized in Table 1; similar recoveries for extraction A were also obtained (results not shown). Except for the initial Sephadex G-15 stages, where labelled folate monoglutamates, relatively low-molecular-weight conjugates and breakdown products of the more labile folate derivatives were filtered out, recovery of radioactivity as metabolites I and II was essentially quantitative and the chromatographic properties of both metabolites remained constant, indicating that little or no decomposition occurred during the purification. Recovery of activity in the microbiological assay during purification did not parallel recovery of radioactivity, activity in the microbiological assay being lost at a higher rate and the ratio of activities in the *L. casei* microbiological assay before and after folate conjugase treatment increased. The latter points suggest that a considerable amount of unlabelled material active in the *L. casei* microbiological assay was present in the original extract. Although a proportion of this activity may be due to the presence of other nutrients concentrated during extraction that could erroneously enhance the growth response, the bulk of this non-radioactive

material active in the *L. casei* microbiological assay was probably unknown folate derivatives.

Comparable amounts of folate conjugate were extracted during extractions A and B (2.36 nmol of folate/g of liver and 2.25 nmol of folate/g of liver respectively by activity in the microbiological assay), demonstrating the reproducibility of extraction.

Confirmation of the amount of folate in the liver as metabolites I and II was obtained by isotope dilution analysis by using the equation:

$$y = \left(\frac{A \cdot a}{A_p} \right) - a$$

where A is the specific radioactivity of the PteGlu administered, a is the total amount of PteGlu administered, A_p is the specific radioactivity of the isolated metabolites (for extraction B these were 2.58 Ci of ^{14}C /mol and 5.54 Ci of ^3H /mol for metabolite I and 2.50 Ci of ^{14}C /mol and 5.95 Ci of ^3H /mol for metabolite II) and y is the total amount of metabolite present assuming 100% incorporation of the radioactivity of the PteGlu. Since the total recovery of radioactivity in metabolites I and II is known (4.13 μCi of ^{14}C and 8.86 μCi of ^3H for metabolite I and 0.50 μCi of ^{14}C and 1.19 μCi of ^3H for metabolite II), the fractional incorporation can be calculated and the total hepatic concentrations of metabolites I and II determined as 1.90 nmol/g of liver (from the ^{14}C radioactivity) and 1.98 nmol/g of liver (from the ^3H radioactivity) for metabolite I and

Table 1. Recoveries of radioactivity and activities in the *L. casei* microbiological assay during the purification of the folate conjugates of rat liver

The folate conjugates in an extract (extraction B) prepared from the livers of 100 rats, some dosed previously with [$2\text{-}^{14}\text{C}$]PteGlu or [$3',5'\text{-Glu-}^3\text{H}$]PteGlu, were purified by column chromatography and the recoveries of radioactivities and activity in the *L. casei* microbiological assay determined. The activity in the *L. casei* microbiological assay determined before (–conjugase) and after (+conjugase) treatment with folate conjugase is expressed as the amount of PteGlu (μg) required for equivalent growth. N.D., Not detected.

Stage of purification	Recovery of radioactivity (%)		Recovery of activity in <i>L. casei</i> microbiological assay (%)	Total activity in <i>L. casei</i> microbiological assay (μg)		Activity ratio (+conjugase/–conjugase)
	^3H	^{14}C		–Conjugase	+Conjugase	
Crude extract	100	100	100	915	3942	4
1st Sephadex G-15 column	73	72	56	343	2202	6
DEAE-cellulose ion-exchange column	70	70	30	29	1179	41
DEAE-Sephadex ion-exchange column						
Metabolite BI	58	59	14	19	533	28
Metabolite BII	8.5	8.7	1.3	4.4	52	12
(Total)	(66.5)	(67.5)	(15.3)	(23.4)	(585)	(25)
Final Sephadex G-15 column						
Metabolite BI	55.1	54.1	16.3	N.D.	641.5	∞
Metabolite BII	7.4	6.6	0.67	N.D.	26.5	∞
(Total)	(62.5)	(60.7)	(17)		(668)	

0.24 nmol/g of liver (from the ^{14}C radioactivity) and 0.25 nmol/g of liver (from the ^3H radioactivity) for metabolite II. The total concentration of these derivatives (metabolite I + metabolite II) found by this method (2.14 nmol/g from the ^{14}C radioactivity and 2.23 nmol/g from the ^3H radioactivity) is thus similar to that determined from the recovery of activity in the microbiological assay. Similar calculations with the data derived from extraction A gave a value of 1.96 nmol/g for the total concentration of folate conjugates (metabolite I + metabolite II). Since the total hepatic folate was about 11 nmol/g (determined by *L. casei* microbiological assay after folate conjugase treatment), the radiolabelled conjugates (metabolite I + metabolite II) make up approx. 20% of the total endogenous hepatic folate.

Physical and chemical properties

The u.v.-absorption and fluorescence spectral data of the major derivative isolated (metabolite I) are summarized in Table 2. In each case the spectra were similar to those obtained for authentic 10-HCO-PteGlu (Table 2) and dissimilar from those of the other biologically important folate derivatives listed by Blakley (1969). However, the chromatographic and electrophoretic behaviour of metabolite I and 10-HCO-PteGlu differ (see Table 4). The u.v.-absorption spectrum of metabolite II could not be determined, as insufficient u.v.-absorbing-impurity-free material was obtained; however, the fluorescence properties were qualitatively similar to those of metabolite I (results not shown). Amino acid analysis of the column-purified metabolite I indicated that only glutamic acid and glycine were present at this stage in the purification, and the latter was lost on subsequent paper chromatography. The final glutamic acid concentration found was 5.4 mol of glutamate/mol of folate (folate determined by *L. casei* microbiological assay) and 4.9 mol of glutamate/mol of folate (folate determined by u.v. absorption). Spectral, t.l.c. and electrophoretic analysis at this stage demonstrated that metabolite I

was an apparently homogeneous single component, and it was thus concluded that metabolite I was a folate tetraglutamate conjugate i.e. contained five glutamate residues.

Enzymic studies

(a) *Comparison of metabolite AI and PteGlu₇*. The distributions of radioactivity among the products of action of intestinal folate conjugase and chicken pancreas folate conjugase are given in Table 3. Rat intestinal folate conjugase hydrolysed PteGlu₇ to PteGlu (>86% of the radioactivity) and the folate conjugate to a derivative that behaved as 10-HCO-PteGlu (>65% radioactivity) in the systems used in analysis. Incubation with chicken pancreas folate conjugase gave different products, and for both substrates these were eluted earlier from Sephadex G-15 columns, i.e. at a lower K_{av} . This suggests that they had higher molecular weights than the PteGlu and 10-HCO-PteGlu produced from the action of rat intestinal folate conjugase and is consistent with the production of pteroyldiglutamate derivatives, PteGlu₂ and 10-HCO-PteGlu₂ as the products of the action of chicken pancreas folate conjugase (Dabrowska *et al.*, 1949). The good yield of PteGlu from PteGlu₇ released by the rat intestinal folate conjugase preparation indicates that little conversion into other folate forms, due to contaminant folate-metabolizing enzymes, occurred during the incubation.

(b) *Product of intestinal folate conjugase action on metabolite BI*. In all systems tested the product of intestinal folate conjugase action on metabolite BI was inseparable from 10-HCO-PteGlu (Table 4). The diversity of the systems used strongly supports the co-identity of the product of intestinal folate conjugase action on metabolite I and 10-HCO-PteGlu. Assuming from the observations above that the activity of any contaminant folate-metabolizing enzymes in the folate conjugase preparation was minimal, then liver metabolite I is a conjugate of 10-HCO-PteGlu.

Table 2. *U.v.-absorption and fluorescence spectra of 10-HCO-PteGlu and the major liver folate conjugate (metabolite I)*
Comparison of the u.v. absorption and the emission and excitation fluorescence spectra of authentic 10-HCO-PteGlu and the major folate conjugate (metabolite I) isolated from rat liver (extraction B) determined at pH 1 (0.1 M-HCl), pH 7 (0.1 M-sodium phosphate buffer) and pH 13 (0.1 M-NaOH).

Derivative	pH	U.v.-absorption $\lambda_{\text{max.}}$ (nm)	Emission		Excitation	
			Excitation λ (nm)	$\lambda_{\text{max.}}$ (nm)	Emission λ (nm)	$\lambda_{\text{max.}}$ (nm)
10-HCO-PteGlu	1	254, 329	320	408	400	320
	7	247, 268, 352	370	458	450	370
	13	259, 372	370	463	450	370
Metabolite I	1	255, 330	320	405	400	320
	7	249, 268, 350	370	455	450	370
	13	261, 365	370	463	450	370

Table 3. Comparison of the products of action of chicken pancreas and rat intestinal folate conjugases on metabolite I and PteGlu₇

Mixtures of ¹⁴C-labelled metabolite I, purified from extracts (extraction A) of the livers of rats some previously dosed with [2-¹⁴C]PteGlu, and authentic [G-³H]PteGlu₇ were incubated with chicken pancreas or rat intestinal folate conjugate preparations, and the products were separated by column chromatography. Rat intestinal folate conjugase hydrolyses folate polyglutamate derivatives to monoglutamate forms and the chicken pancreas enzyme hydrolyses folate polyglutamates to diglutamate derivatives (Dabrowska *et al.*, 1949). Values quoted are the percentage radioactivities in the products resolved by Sephadex G-15 gel filtration. N.D., Not detected.

Enzyme treatment	K _{av} Identity	³ H-labelled products derived from PteGlu ₇			¹⁴ C-labelled products derived from metabolite I		
		0.06 PteGlu ₇	0.90 PteGlu ₂	1.57 PteGlu	0.18 Metabolite I	0.48 10-HCO-PteGlu ₂	0.78 10-HCO-PteGlu
Chicken pancreas folate conjugase		6	64	4	13	58	4
Rat intestinal folate conjugase		4	6	86	14	11	65
None		70	17	N.D.	80	8	N.D.

Table 4. Summary of the chromatographic properties of metabolite I, of its rat intestinal folate conjugase hydrolysis product and of authentic 10-HCO-PteGlu

The chromatographic and electrophoretic properties of metabolite I, of the product of rat intestinal folate conjugase action on metabolite I and of authentic 10-HCO-PteGlu are compared. In all cases the product of rat intestinal folate conjugase action on metabolite I behaved similarly to 10-HCO-PteGlu, indicating that metabolite I is a 10-HCO-PteGlu conjugate. 10-HCO-PteGlu was detected by its fluorescence and u.v. absorption; metabolite I and its folate conjugase hydrolysis product were detected by radioactivity measurements (A), by fluorescence (B) or by bioautography (C).

Chromatographic system	Method of detection	Metabolite I	Metabolite I after conjugase treatment	10-HCO-PteGlu
DEAE-cellulose (molarity of NaCl)	A	0.55	0.50	0.50
Sephadex G-15 gel filtration (K _{av})	A	0.18	0.72	0.72
T.l.c. (R _F values)				
(1) Propan-1-ol/aq. NH ₃ (sp.gr. 0.880)/water (200:1:199) (by vol.)	A, B	0.08	0.31	0.31
(2) Aq. 3% (w/v) NH ₄ Cl	A, B	0.96	0.68	0.66
(3) 0.05 M-Sodium phosphate buffer, pH 7	A, B	—	0.71	0.71
Thin-layer gel filtration (migration relative to Dextran Blue)	B, C	0.94	0.39	0.39
Electrophoresis (migration relative to PteGlu)	B, C	1.56	0.72	0.72

Differential microbiological assay

Metabolites I and II were inactive in the microbiological assay without prior folate conjugase treatment. Folate conjugase action released material with maximal activities in the *L. casei* and *S. faecalis* microbiological assays but no detectable activity in the *P. cerevisiae* microbiological assay from both metabolites. This is consistent with a 10-HCO-PteGlu conjugate identity for both metabolites (see Blakley, 1969).

Discussion

The principal radiolabelled folate derivative found in rat liver 48 h after administration of PteGlu has been shown to be a pteroylpentaglutamate conjugate. Spectral analysis of the conjugate and a detailed chromatographic, electrophoretic and

microbiological-activity investigation of the product of folate conjugase action on the conjugate identified the metabolite as a 10-HCO-PteGlu derivative. The proposed structure of the metabolite, 10-HCO-PteGlu₃, is illustrated in Fig. 1. The structure incorporates the γ -linked glutamate chain often ascribed to folate conjugates. The structure of the peptide chain of naturally occurring folate polyglutamates has never been determined absolutely. Folate conjugates were originally attributed with the unique γ -linkage from the observed properties of folate conjugase preparations, since folate conjugase enzymes were shown to hydrolyse synthetic γ -glutamyl derivatives at a much higher rate than synthetic α -glutamyl derivatives (Kazenko & Laskowski, 1948; Dabrowska *et al.*, 1949). In the present study the liver folate conjugates were shown to be substrates for preparations of folate conjugases, preparations

Fig. 1. Structure of 10-formylfolylpolyglutamate derivatives
10-HCO-PteGlu, $n = 0$; metabolite I (10-HCO-PteGlu₃), $n = 4$; metabolite II (10-HCO-PteGlu₄), $n = 3$.

that also hydrolysed authentic PteGlu₇, a chemically synthesized folate conjugate containing the γ -linkage in the peptide chain. Although contamination of the rat intestinal folate conjugase with non-specific carboxypeptidases would explain the concomitant release of 10-HCO-PteGlu from 10-HCO-PteGlu₄ and of PteGlu from PteGlu₇, even if the former conjugate contained α - not γ -linked glutamate residues, this is not so for the chicken pancreas enzyme, since the products obtained from both the natural and synthetic conjugates behaved as pteroyldiglutamate not pteroylmonoglutamate derivatives and therefore were the products of a specific enzyme activity. This evidence suggests that the amide bond between the first two glutamate residues, and probably the remaining amide bonds, is the same for both the liver folate conjugate and that of PteGlu₇, and thus the liver polyglutamates isolated contain the γ -glutamyl linkage.

The minor derivative metabolite II was present in liver in amounts of about 12% that of 10-HCO-PteGlu₃. Because of its lower concentration it was not possible to characterize this derivative extensively. Since the compound was active in the *L. casei* microbiological assay only after folate conjugase treatment, it probably contained more than three glutamate residues/molecule as *L. casei* responds to folate conjugates containing up to three glutamate residues/molecule without prior folate conjugase treatment (Blakley, 1969). The fluorescence properties of metabolite II and of 10-HCO-PteGlu₃ were similar, as were the activities in the differential microbiological assays. The specific radioactivities found for metabolites I and II were almost identical, suggesting that they were very closely related, one probably being derived directly from the other. On Sephadex G-15 gel filtration metabolite II was eluted slightly later than was metabolite I, suggesting that it had a lower molecular weight. It is therefore proposed that metabolite II is 10-HCO-PteGlu₄.

10-HCO-PteGlu₃ has also been isolated from *Escherichia coli* (Friedkin *et al.*, 1975), where it was characterized as a potent inhibitor of mammalian dihydrofolate reductase. d'Urso-Scott *et al.* (1974) reported that, on incubating rat liver slices with PteGlu₃, 10-HCO-PteGlu₃ accumulated. Since this formylation reaction proceeded even in the presence of the dihydrofolate reductase inhibitor methotrexate, they concluded that a specific synthesis of 10-HCO-PteGlu₃ that did not involve a reduced folate intermediate was occurring and that this reaction possibly fulfilled a regulatory role *in vivo*. Such a function would seem to be even more suited to 10-HCO-PteGlu₃, since this is a more powerful inhibitor of mammalian dihydrofolate reductase than is 10-HCO-PteGlu (Friedkin *et al.*, 1975). However, since the relatively stable compound 10-HCO-PteGlu is very readily produced by the oxidation of 10-HCO-H₄PteGlu (Robinson, 1971; Maruyama *et al.*, 1978), an important reduced coenzyme form, it is possible that the 10-HCO-PteGlu₃ isolated was largely derived from 10-HCO-H₄PteGlu₃ during extraction.

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