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The Synthesis of Pteroylpolyglutamates by Sheep Liver Enzymes *in vitro*

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1. Sephadex G-15 was used to separate pteroylmonoglutamates from corresponding polyglutamate derivatives. 2. Pteroylpolyglutamates were formed when 5-formyltetrahydro[2-^{14}C]pteroylglutamic acid, 5-[*methyl*-^{14}C]tetrahydropteroylglutamic acid or tetrahydro[2-^{14}C]pteroylglutamic acid was incubated at pH 8.4 with ATP, $MgCl_2$, KCl, L-glutamic acid and sheep liver cytosol. The γ-glutamyl side chain appeared to be lengthened by the stepwise addition of single glutamate moieties. 3. The subcellular distribution of pteroylpolyglutamates paralleled that of pteroylpolyglutamate synthetase activity, and followed the order cytosol > 'nuclear' fraction > microsomal fraction > mitochondria.

From 75 to 99% of the folate in the liver of animals fed on a nutritionally adequate diet is in the form of pteroylpolyglutamates (Bird *et al.*, 1965; Osborne-White & Smith, 1973). These compounds are considered to be storage forms of the metabolically active tetrahydropteroylmonoglutamates, and are formed from them by the addition of glutamic acid molecules in γ-peptide linkage.

The lack of a suitable system for observing the enzymic conversion of pteroylmonoglutamates into polyglutamates *in vitro* has hampered investigations into the nature of the reaction, and into its role in metabolic disturbances, such as vitamin B_{12} deficiency, where tissue contents of pteroylpolyglutamates decrease dramatically (Kutzbach *et al.*, 1967; Smith & Osborne-White, 1973).

In the present paper we describe the synthesis of pteroylpolyglutamates *in vitro* in a system that requires ATP, Mg^{2+}, K^+, L-glutamic acid, a reduced pteroylmonoglutamate and enzyme from sheep liver. The conditions for optimum rates of synthesis and the intracellular distribution of enzymes catalysing the synthesis are described.

Experimental

Animals

Adult Australian Merino ewes were housed in pens and fed *ad libitum* on a diet of equal parts by weight of dried lucerne-chaff and wheaten hay-chaff. Free access to food and water was permitted until the animals were killed by cutting the throat.

Materials

*Pteroylglutamic acid derivatives.** [2-^{14}C]Pteroylglutamic acid (specific radioactivity 55.3 mCi/mmol) and (±)5-[*methyl*-^{14}C]tetrahydropteroylglutamate (barium salt, specific radioactivity 61 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The radioactive methyltetrahydropteroylglutamate was converted into the free acid by passage through Dowex 50W (X4, H^+ form, 50–100 mesh) and was purified by column chromatography on DEAE-Sephadex at 0–2°C, with a linear gradient (0–0.8 M) of ammonium acetate containing 0.2% (w/v) 2-mercaptoethanol. The major elution peak of radioactive 5-CH_3-H_4PteGlu was pooled, and ammonium acetate was removed by freeze-drying. Because there was some decomposition during this process, it was difficult to obtain preparations greater than 95% radiochemically pure.

(±)5-HCO-H_4[2-^{14}C]PteGlu was prepared from [2-^{14}C]PteGlu by formylation in 98% (v/v) formic acid (Blakley, 1959), reduction with $NaBH_4$ (Scrimgeour & Vitols, 1966), and isomerization at pH 7.4 and 120°C for 1 h. The product was purified on DEAE-Sephadex and isolated from the eluate in a

* Abbreviations: PteGlu, pteroyl-L-glutamic acid; H_4PteGlu; 5,6,7,8-tetrahydropteroyl-L-glutamic acid; 5-HCO-H_4PteGlu, 5-formyltetrahydropteroyl-L-glutamic acid; 5-CH_3-H_4PteGlu, 5-methyltetrahydropteroyl-L-glutamic acid. Forms with additional conjugated γ-glutamyl groups are numbered with a subscript indicating the total number of glutamate residues in the molecule, as set out in IUPAC–IUB tentative rules, *Biochem. J.* (1967) **102**, 19–20.

manner similar to that described for 5-[*methyl*-^{14}C]-H_4PteGlu.

Purified 5-HCO-H_4[2-^{14}C]PteGlu and 5-[*methyl*-^{14}C]H_4PteGlu were each dissolved in 10mM-Tris–HCl, pH7.0 and stored frozen.

(±)Tetrahydro[2-^{14}C]pteroylglutamic acid was prepared from [2-^{14}C]PteGlu by catalytic hydrogenation in 0.1M-potassium phosphate buffer, pH7.0,

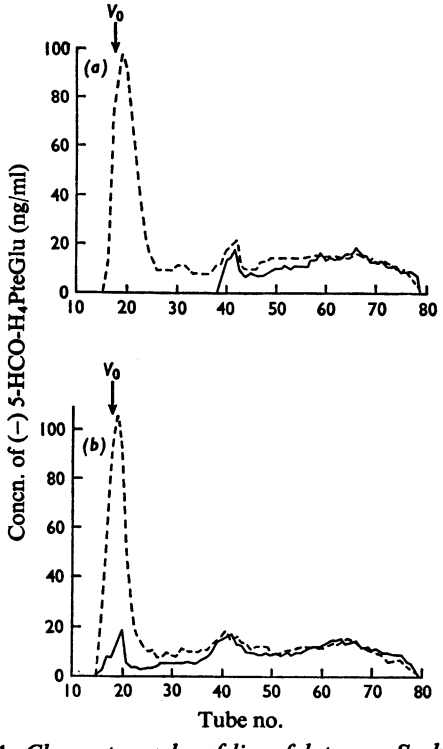


Fig. 1. *Chromatography of liver folates on Sephadex G-10*

Liver (10g) from a rat fed on a stock diet was extracted at pH6.0 and 95°C for 10min in the presence of 1% ascorbate. After centrifugation, the supernatant was diluted to 5ml with 1% ascorbate, pH6.0 and 1ml was applied to the top of a column (1.5cm×88cm) of Sephadex G-10. The column was eluted at 4°C with 1M-potassium phosphate, pH6.3, 1% ascorbate, at a flow rate of 7ml/h. The contents of each tube was analysed for folate by microbiological assay with (*a*) *S. faecalis* and (*b*) *L. casei*, both before (——), and after (----) treatment with hog-kidney conjugase (Bird *et al.*, 1965). The folate concentration in the eluate is expressed as ng of folate activity for the assay organisms, based on a standard of (–)5-HCO-H_4PteGlu. V_0 was the void volume (Dextran Blue 2000), and each tube contained 3.0ml of eluate.

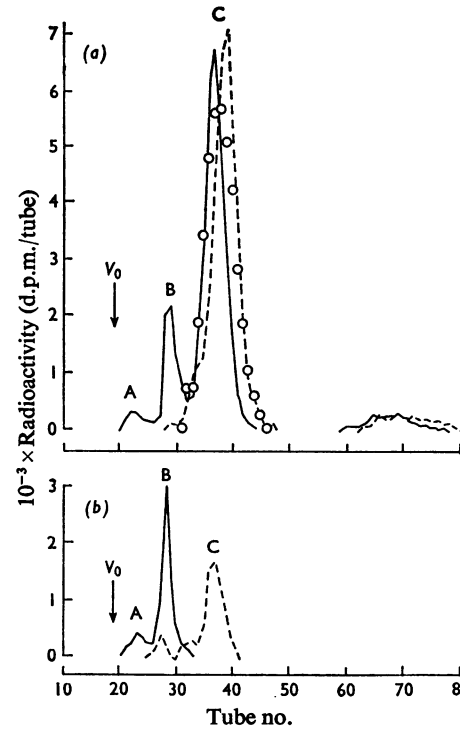


Fig. 2. *Synthesis of polyglutamate forms of 5-HCO-H_4[2-^{14}C]PteGlu*

(*a*) (±) 5-HCO-H_4[2-^{14}C]PteGlu (2.4nmol; 55.3mCi/mmol) was incubated at 37°C with 12μmol of ATP, 24μmol of $MgCl_2$, 360μmol of KCl, 24μmol of L-glutamate, 120μmol of Tris–HCl (pH8.4) and 1.2ml of 1:5 sheep liver cytosol. The volume of the reaction mixture was 2.4ml. After 1h of incubation, 0.3ml of 10% ascorbate, pH6.0, was added and the reaction mixture was heated at 95°C for 10min, then centrifuged. Supernatant (1ml) was chromatographed on a column (1.32cm×150cm) of Sephadex G-15 (——). Another 1ml portion was treated with hog kidney conjugase before chromatography on a similar column (----). (±) 5-HCO-H_4[2-^{14}C]PteGlu incubated under comparable conditions, omitting sheep liver cytosol, was eluted in the peak marked by (○). (*b*) 5-HCO-H_4[2-^{14}C]PteGlu was incubated with sheep liver cytosol and other compounds as described above, and 2ml of a 1% ascorbate extract of the incubation was applied to Sephadex G-15 and eluted with 0.1M-ammonium acetate containing 0.2% (w/v) mercaptoethanol. The first two radioactive peaks (A and B) were pooled, and concentrated by freeze-drying. Half of the freeze-dried product was dissolved in 1% ascorbate and re-chromatographed on Sephadex G-15 (——), the other half was treated with hog kidney conjugase before re-chromatography (----). V_0 was the void volume (Dextran Blue 2000). Each tube contained 2.9ml of eluate.

with platinum oxide catalyst, and was used directly, without further treatment.

Chemicals. KCl, $MgCl_2$, glutamic acid, glutamine, ascorbic acid and formic acid were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. ATP and 2-mercaptoethanol were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sephadex G-10, G-15 and DEAE-Sephadex A-25 were obtained from Pharmacia, Uppsala, Sweden, diethanolamine was obtained from L. Light and Co. Ltd., Colnbrook, Bucks., U.K., and sucrose was obtained from the Colonial Sugar Refining Co. Ltd., Sydney, Australia.

Methods

Incubations of liver homogenate with radioactive pteroylmonoglutamate, ATP, $MgCl_2$, KCl and potassium glutamate were carried out *in vitro* in stoppered glass vessels at 37°C. After incubating for the desired time, ascorbate, pH 6.0, was added to a final concentration of 1%, and the mixture was then heated at 95°C for 10min to terminate the reaction and extract the folates. The denatured proteins were removed by centrifugation, and 0.5–1.0ml of supernatant was applied to the top of a column (1.32cm×150cm) of Sephadex G-15. The column was eluted with 0.1M-NaCl containing 0.2% mercaptoethanol, at a constant flow rate of 200ml/24h, in a cold room at 0–2°C.

Fractions of 3ml to 5ml volume were collected, and 1ml of each fraction was counted in a Packard Model 3375 scintillation counter with a 6:7 (v/v) Triton X-100–toluene scintillant (Patterson & Greene, 1965). Quenching corrections were made by the external standardization method. The counting efficiency $\left(\frac{\text{nett c.p.m.}}{\text{d.p.m.}} \times 100\right)$ was 40–60%.

Subcellular fractionation of liver. Liver (100g) from freshly killed animals was chilled and homogenized (30g of liver mash plus 120ml of 0.25M-sucrose) by the procedure described by Smith *et al.* (1965). The homogenate (whole homogenate) was then divided, by centrifugal fractionation at 2°C (Hogeboom *et al.*, 1948), into the following fractions: (i) 'nuclear' fraction, consisting of nuclei, unbroken cells and cell debri (600*g* sediment, 10min in MSE Highspeed 18 centrifuge) (ii) nuclear-free homogenate (600*g* supernatant) (iii) mitochondria, twice washed with 0.25M-sucrose (6600*g* sediment 10min in a Spinco

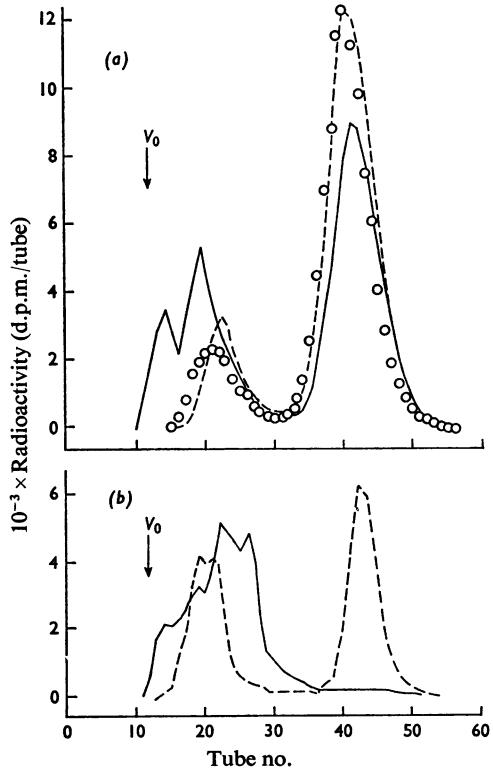


Fig. 3. *Synthesis of polyglutamate forms of 5-[methyl-^{14}C]-H_4PteGlu*

(*a*) (±) 5-[*methyl*-^{14}C]H_4PteGlu (12nmol; 61mCi/mmol) was incubated at 37°C with 40μmol of ATP, 80μmol of $MgCl_2$, 960μmol of KCl, 120μmol of L-glutamate, 800μmol of Tris–HCl (pH 8.4) and 4ml of 1:5 sheep liver cytosol. The volume of the reaction mixture was 8ml. After 2h of incubation the pH was adjusted to 6.0, and 1.0ml of 10% ascorbate, pH 6.0, was added. The mixture was heated at 95°C for 10min, then centrifuged. Supernatant (1ml) was chromatographed on a column (1.32cm×150cm) of Sephadex G-15 (——). Another 1ml portion was treated with hog kidney conjugase before chromatography on a similar column (----). 5-[*methyl*-^{14}C]-H_4PteGlu incubated under similar conditions but omitting sheep liver cytosol, was eluted in the peaks marked by (○). Note the breakdown product of 5-[*methyl*-^{14}C]H_4PteGlu appearing in tubes 15–30. (*b*) 5-[*methyl*-^{14}C]H_4PteGlu was incubated with sheep liver cytosol and other compounds as described above, and 4ml of a 1% ascorbate extract of the incubation was applied to Sephadex G-15 and eluted with 0.1M-ammonium acetate containing 0.2% (w/v) mercaptoethanol. The first two radioactive peaks were pooled and concentrated by freeze drying. Half of the freeze-dried product was dissolved in 1% ascorbate and re-chromatographed on Sephadex G-15 (——) and the other half was treated with hog kidney conjugase before re-chromatography (----). V_0 was the void volume (Dextran Blue 2000), and each tube contained 4.2ml of eluate.

model L preparative ultracentrifuge) (iv) mitochondrial supernatant (6600*g* supernatant) (v) microsomal fraction (microsomes) (105500*g* sediment, 60min in a Spinco model L preparative ultracentrifuge) (vi) cytosol (105500*g* supernatant).

Folate assay. Samples that had been extracted with 1% ascorbate, pH6.0, at 95°C for 10min were analysed for folate before and after hog kidney conjugase treatment (Bird *et al.*, 1965) by microbiological assay with *Lactobacillus casei* or *Streptococcus faecalis.*

Protein. This was measured by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (Sigma Chemical Co.) as the standard.

Results and Discussion

Chromatography of liver folates on Sephadex

Preparations of hog kidney conjugase contain the enzyme γ-glutamylcarboxypeptidase which specifically hydrolyses the peptide linkages in pteroylpolyglutamates to leave only one glutamate residue per molecule (Blakley, 1969; Bolinder *et al.*, 1953). Because the micro-organisms commonly used for the assay of folates do not respond to pteroylpolyglutamates with more than three glutamate residues per molecule (Stokstad & Koch, 1967), it is necessary to use γ-glutamylcarboxypeptidase to remove the extra glutamate moieties from these compounds before they can be detected microbiologically.

When an ascorbate extract of liver (Bird *et al.*, 1965) was chromatographed on Sephadex G-10 or G-15, most of the folate that appeared early in the elution profile was unavailable for the growth of *L. casei* or *S. faecalis* unless it had been treated with hog kidney conjugase, whereas the compounds that eluted later were directly available (Fig. 1). These results suggested that Sephadex could be used to separate the larger pteroylpolyglutamates from the unconjugated derivatives, and could therefore be applied to the detection of newly synthesized polyglutamates in incubations *in vitro*. The chromatographic separation is only partly effected by exclusion chromatography and is apparently aided by adsorptive interactions with the gel that delay the elution of unconjugated folates beyond that expected on the basis of molecular size alone.

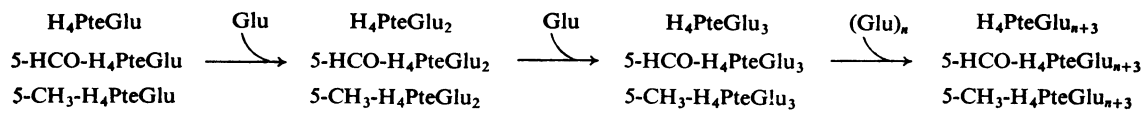
Pteroylpolyglutamate synthesis in vitro

When ascorbate extracts of incubations that had contained 5-HCO-H_4[2-^{14}C]PteGlu, ATP, $MgCl_2$,

Table 1. *Requirements of pteroylpolyglutamate synthesis by sheep liver enzymes in vitro*

The complete reaction mixture contained 0.64nmol of (±)5-HCO-H_4[2-^{14}C]PteGlu, or 0.59nmol of (±)5-[*methyl*-^{14}C]H_4PteGlu, or 0.61nmol of (±)H_4[2-^{14}C]PteGlu, and 2.5μmol of ATP, 5μmol of $MgCl_2$, 75μmol of KCl, 5μmol of L-glutamic acid, 50μmol of Tris–HCl (pH8.4) and 0.25ml of 1:5 sheep liver cytosol. The volume of reaction mixture was 0.5ml. After 30min reaction at 37°C, 0.1ml of 6% ascorbate, pH6.0, was added to each incubation and the mixture was heated for 10min at 95°C. The ascorbate extracts were centrifuged and 0.3ml of supernatant was chromatographed on Sephadex G-15. The quantity of pteroylpolyglutamate synthesized was calculated as described in the text. The results are expressed as pmol of pteroylpolyglutamate formed/mg of protein in 30min. n.d. denotes <0.7pmol/mg of protein in 30min.

	Pteroylmonoglutamate substrate		
Reaction mixture	5-HCO-H_4[2-^{14}C]PteGlu	5-[*methyl*-^{14}C]H_4PteGlu	H_4[2-^{14}C]PteGlu
Complete	9.8	12.6	24.6
Omit ATP	n.d.	n.d.	n.d.
Omit $MgCl_2$	n.d.	n.d.	n.d.
Omit KCl	1.4	3.6	2.5
Omit L-glutamic acid	1.1	0.7	4.7
Omit cytosol	n.d.	n.d.	n.d



Scheme 1. *Scheme for the conversion of pteroylmonoglutamates into polyglutamates*

KCl, L-glutamate and sheep liver cytosol were chromatographed on Sephadex G-15, two early-eluting peaks of radioactivity appeared (peaks A and B, Fig. 2*a*). These peaks were not detected if the extracts were treated with γ-glutamylcarboxypeptidase before Sephadex chromatography (Fig. 2*a*), indicating that they were polyglutamate forms of 5-HCO-H_4-[2-^{14}C]PteGlu. This was confirmed by isolating peaks A and B free of unchanged 5-HCO-H_4[2-^{14}C]PteGlu and then rechromatographing the isolate before and after γ-glutamylcarboxypeptidase treatment (Fig. 2*b*). Other evidence consistent with peaks A and B being pteroylpolyglutamates is that: (*a*) their formation is dependent on the presence of glutamic acid (Table 1) (*b*) they elute in a position similar to pteroylpolyglutamates synthesized *in vivo* (Fig. 1).

Comparable results were obtained when 5-[*methyl*-^{14}C]H_4PteGlu or H_4[2-^{14}C]PteGlu were used as substrates. With 5-[*methyl*-^{14}C]H_4PteGlu a significant proportion of the radioactivity that eluted in the position of polyglutamates (between tubes 10 and 30, Fig. 3) was contributed by decomposition products of the substrate. The elution behaviour of these compounds was unaffected by γ-glutamylcarboxypeptidase treatment (Fig. 3). In calculating the quantity of polyglutamate synthesized, allowance was made for radioactivity from this source by chromatographing extracts of control incubations that contained all factors except cytosol.

The quantity of pteroylpolyglutamate synthesized in the incubations *in vitro* was calculated from the amount of radioactivity in the relevant peaks eluted from Sephadex G-15 as follows:

$$\frac{\text{c.p.m. pteroylpolyglutamate}}{\text{counting efficiency}} \times \frac{\text{total vol. of ascorbate extract}}{\text{vol. of extract chromatographed}} \times \frac{100}{\text{specific activity of added pteroylmonoglutamate}}$$

The variable, but negligible, concentrations of pteroylmonoglutamate contributed by the cytosol (<0.01 μM) was ignored in the calculation of the specific activity of the pteroylmonoglutamate substrates.

Only ATP, $MgCl_2$, KCl and L-glutamate were required in addition to the pteroylmonoglutamates for the production of pteroylpolyglutamates *in vitro* (Table 1). GTP could not be substituted for ATP, and glutamine, glutathione or γ-glutamylglutamate could not be substituted for L-glutamate. The pteroylpolyglutamates appeared to be formed from the corresponding pteroylmonoglutamates by stepwise addition of single glutamate molecules as shown in Scheme 1.

The only previous report of enzymic synthesis of pteroylpolyglutamates *in vitro* known to us is that of Griffin & Brown (1964). These workers tentatively established that $H_4PteGlu_2$ and $H_4PteGlu_3$ were formed from H_4PteGlu in the presence of ATP, $MgCl_2$, L-glutamate and the 0–63% $(NH_4)_2SO_4$ precipitate of charcoal-treated *Escherichia coli* extract. Under these conditions neither 5-HCO-H_4PteGlu nor 5-CH_3-H_4PteGlu could replace H_4-PteGlu as a substrate 'for the formation of a new compound active as a growth factor for *L. casei*.' The authors implied that the *E. coli* enzyme could not catalyse the conversion of 5-HCO-H_4PteGlu or 5-CH_3-H_4PteGlu into polyglutamates. In contrast, the sheep liver enzymes used in our experiments did catalyse these conversions.

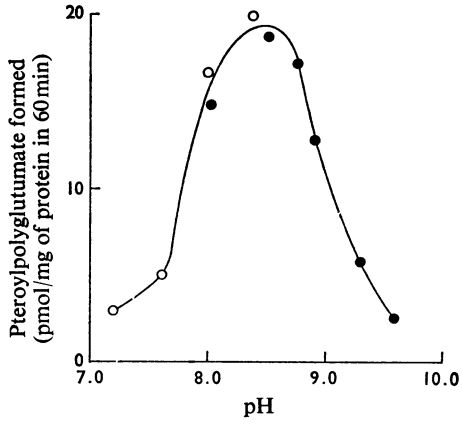


Fig. 4. *Effect of pH on pteroylpolyglutamate synthesis*

(±) 5-HCO-H_4[2-^{14}C]PteGlu (1.45nmol; 55.3mCi/mmol), 10μmol of ATP, 20μmol of $MgCl_2$, 300μmol of KCl, 20μmol of L-glutamate and 1ml of 1:5 sheep liver cytosol were incubated at several pH values controlled by 0.1M-Tris–HCl (○) or 0.1M-diethanolamine–HCl (●) buffer. The reaction was carried out at 37°C in a total volume of 2ml. After 1h of incubation, 0.3ml of 10% ascorbate, pH6.0, was added and the mixture was heated at 95°C for 10min, then centrifuged. Supernatant (0.5ml) was chromatographed on a column (1.32cm×150cm) of Sephadex G-15. The rate of pteroylpolyglutamate synthesis was calculated from the size of eluted peaks, as described in the text.

Optimum conditions for pteroylpolyglutamate synthesis in vitro

The optimum pH for the reaction was between 8.3 and 8.4 (Fig. 4).

The rates of synthesis of pteroylpolyglutamates at different concentrations of L-glutamate, KCl and

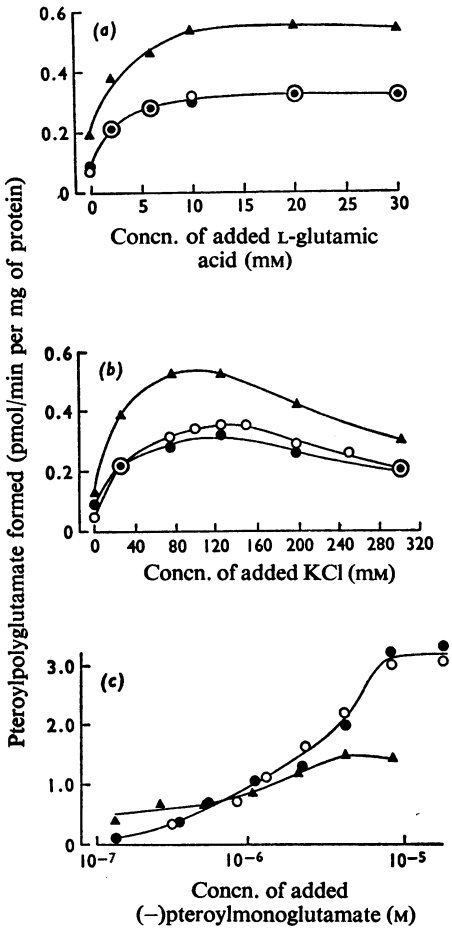


Fig. 5. *Effect of different concentrations of glutamic acid, KCl and pteroylmonoglutamate on the rate of pteroylpolyglutamate synthesis*

(*a*) (±)5-HCO-H_4[2-^{14}C]PteGlu (0.73nmol; 55.3mCi/mmol) (○), (±)5-[*methyl*-^{14}C]-H_4PteGlu (0.66nmol; 61.0mCi/mmol) (●), or (±)H_4[2-^{14}C]PteGlu (0.73nmol; 55.3mCi/mmol) (▲), was incubated with 5μmol of ATP, 10μmol of $MgCl_2$, 150μmol of KCl, 100μmol of Tris–HCl (pH8.4), 0.5ml of 1:5 sheep liver cytosol and several concentrations of L-glutamate at 37°C for 30min. The volume of the reaction mixture was 1.0ml. The reaction was stopped by adding 0.1ml of 10% ascorbate, pH6.0, and heating at 95°C for 10min. After centrifugation, 0.5ml of supernatant was chromatographed on Sephadex G-15, and the quantity of pteroylpolyglutamate synthesized was calculated as described in the text. (*b*) Similar incubations were carried out as in (*a*), except that the rate of pteroylpolyglutamate synthesis was measured at several concentrations of KCl, in the presence of 10mM-L-glutamate. (*c*) The rate of pteroylpolyglutamate synthesis at different concentrations of 5-HCO-H_4PteGlu, 5-CH_3-H_4-PteGlu or H_4PteGlu was measured in 0.5ml incubations containing 2.5μmol of ATP, 5μmol of $MgCl_2$, 75μmol of KCl, 5μmol of L-glutamate, 50μmol of Tris–HCl (pH8.4) and 0.25ml of 1:5 sheep liver cytosol. The reaction mixtures were incubated for 15min at 37°C, extracted with 1% ascorbate at pH6.0 and chromatographed on Sephadex G-15. The rate of pteroylpolyglutamate synthesis was calculated as described in the text. The concentration of biologically active (−) isomers of the pteroylmonoglutamates (50% of the concentration of the chemically synthesized compounds) is shown as the abscissa.

Table 2. *Subcellular distribution of pteroylpolyglutamate synthetase activity*

Each incubation contained 1.34nmol of (±) 5-HCO-H_4[2-^{14}C]PteGlu, or 1.43nmol of (±) 5-[*methyl*-^{14}C]H_4PteGlu or 1.38nmol of H_4[2-^{14}C]PteGlu together with 10μmol of ATP, 20μmol of $MgCl_2$, 300μmol of KCl, 20μmol of L-glutamate and 1.0ml of the appropriate sub-fraction of 1:5 sheep liver homogenate (see text). The reaction volume was 2.0ml. After 30min of reaction at 37°C, 0.3ml of 10% ascorbate, pH6.0, was added to each incubation and the folates were extracted at 95°C for 10min. Extracts were centrifuged and 0.5ml portions of supernatant were chromatographed on Sephadex G-15. The quantity of pteroylpolyglutamate synthesized was calculated as described in the text. The results are expressed as pmol of pteroylpolyglutamate synthesized/mg of protein in 30min.

	Pteroylmonoglutamate substrate		
Liver fraction	5-HCO-H_4[2-^{14}C]PteGlu	5-[*methyl*-^{14}C]H_4PteGlu	H_4[2-^{14}C]PteGlu
Whole homogenate	3.5	4.7	1.7
'Nuclear' fraction	2.9	7.2	6.2
Mitochondria	<2.0	1.2	<4.8
Microsomes	1.8	7.6	<2.5
Cytosol	8.7	13.0	14.7
Mitochondrial supernatant	3.8	8.2	2.2
Nuclear-free homogenate	4.7	6.4	2.7

Table 3. *Subcellular distribution of folates in sheep liver*

The table shows a typical sub-cellular distribution of folates in the liver of penned adult sheep fed *ad libitum* on a diet of equal parts by weight of wheaten hay-chaff and dried lucerne-chaff. Freshly excised liver was homogenized in cold 0.25M-sucrose and fractionated by differential centrifugation (see the text). The folate content of each fraction was extracted in the presence of 1% ascorbate, pH6.0, for 10min at 95°C. The extracts were centrifuged and the supernatants were analysed for folate by microbiological assay with *L. casei* before and after hog kidney conjugase treatment (Bird *et al.*, 1965). The folate content per g wet wt. of liver, or per mg of protein of each sub-cellular fraction is expressed as μg or pmol of folate activity for *L. casei* based on a standard of (−)5-HCO-H_4PteGlu. The 'larger' pteroylpolyglutamates are those that were unavailable to *L. casei* without prior conjugase treatment.

	(*a*) Before conjugase treatment		(*b*) After conjugase treatment		Larger pteroylpolyglutamates [(*b*)−(*a*)]	
Liver	(μg/g of liver)	(pmol/mg of protein)	(μg/g of liver)	(pmol/mg of protein)	(μg/g of liver)	(pmol/mg of protein)
Whole homogenate	0.11	1.31	9.84	101.75	9.73	100.44
'Nuclear' fraction	0.01	0.35	1.20	44.83	1.19	44.48
Mitochondria	0.001	0.22	0.08	12.77	0.079	12.55
Microsomes	0.01	0.24	0.48	24.40	0.47	24.16
Cytosol	0.08	1.95	7.92	206.37	7.84	204.42

pteroylmonoglutamate substrate are shown in Fig. 5. The rate of synthesis was stimulated by increasing the added concentration of KCl to 120mM, but above 150mM the rate declined. The synthesis that occurred in the absence of added glutamate was probably due to the small amount of this substance contained in liver cytosol.

Maximum rates of synthesis were obtained with pH8.4 Tris–HCl buffer, 120mM-KCl, 15mM-L-glutamate, 5mM-ATP, 10mM-$MgCl_2$, and 60μM-(±) pteroylmonoglutamate substrate.

Sub-cellular distribution of pteroylpolyglutamate synthetase

The sub-cellular distribution of sheep liver pteroylpolyglutamate synthetase activity is shown in Table 2. With each of the three substrates, the synthetase activity was greatest in cytosol, followed in decreasing order by the 'nuclear' fraction, microsomes and mitochondria. Because the 'nuclear' fraction contained an appreciable proportion of whole cells, cell debris and erythrocytes, results with this fraction cannot be attributed solely to nuclei.

Table 3 shows the subcellular distribution of folates in the liver of healthy sheep. Almost all (>98%) of the folate in each fraction was in the form of pteroylpolyglutamates. By comparing Table 3 with Table 2 it is clear that the subcellular distribution of pteroylpolyglutamates paralleled that of the activity of the enzymes that catalysed their synthesis.

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