Identification and Measurement of the Folates in Sheep Liver

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1. Methods are described for the extraction, separation by ion-exchange chromatography and estimation by microbiological assay of the folates in sheep liver. 2. Injection of [2-¹⁴C]-pteroylglutamate into a sheep fed on a stock diet led to extensive labelling of chromatographically separable liver folates. About 12% of the label in the liver could not be extracted by the method used. 3. Liver folates were examined in five ewes fed on restricted amounts of a diet of wheaten hay-chaff and gluten and injected weekly with vitamin B₁₂. Chromatographic separation was followed by microbiological assay with *Lactobacillus casei*, *Streptococcus faecalis* R. and *Pediococcus cerevisiae* both before and after treatment of fractions with conjugase (γ -glutamylcarboxypeptidase). Evidence was obtained that the folates present were predominantly polyglutamate forms of tetrahydropteroylglutamate, 5-methyltetrahydropteroylglutamate and 5- (and 10-) formyltetrahydropteroylglutamates. Differences in the responses of the assay organisms permitted quantitative distinction between these three main classes of folates. 4. Methyltetrahydrofolates were eluted in seven successive peaks that were separated by constant increments in the logarithm of eluant [P₁]. A similar relationship existed for seven successive peaks of tetrahydrofolate and may also have existed for each of the two series of formyltetrahydrofolates. 5. Based on these and other observations it is proposed that sheep liver folates consist predominantly of the mono- to hepta-glutamates of each of the reduced pterates identified. The methods employed allowed quantitative determinations to be made of most of the folates present. The predominant forms were hexaglutamates. 6. Four components active for *L. casei* were detected that could not be identified. Three of them were polyglutamates.

Pteroylpolyglutamates occur throughout the evolutionary scale (Blakley, 1969, pp. 25-49) perhaps mainly as storage forms. In only a few rather unusual instances have functions for them been found that were not fulfilled as well, or better, by the corresponding monoglutamates (Wright, 1956; Blakley, 1957; Guest & Jones, 1960; Kozloff *et al.*, 1970). Because of technical limitations, however, this topic has not been systematically studied. Methods for the synthesis of pteroylpolyglutamates (γ -L-glutamyl peptides of pteric acid) have only recently been developed (Krumdieck & Baugh, 1969; Godwin *et al.*, 1972) and until the present time there has been no completely satisfactory analytical method for the resolution of the natural forms present in tissues.

Most attempts to separate natural folates have been based on the method of Silverman *et al.* (1961) in which a phosphate gradient was used to elute folates adsorbed on DEAE-cellulose. Perhaps the most successful application of this method was made by Shin *et al.* (1972) who separated ³H-labelled folates from rat liver into ten distinct radioactive peaks. Many of these peaks corresponded to the elution positions of two or more folates but by a combination of molecular-sieve chromatography, differential

microbiological activity and comparison with synthetic pteroylpolyglutamates Shin *et al.* (1972) were able to suggest the presence of a number of specific forms. According to their analysis 85-90% of the folates from rat liver were reduced pteroylpenta-glutamates. Monoglutamates accounted for 5% of total folates and the remainder were di- or tri-glutamates. No tetraglutamates were detected and tetrahydropteroyltriglutamate (H₄PteGlu₃*) was also absent. The forms identified were H₄PteGlu_n, 5-CH₃-H₄PteGlu_n; 5-HCO-H₄PteGlu_n and 10-HCO-H₄PteGlu_n but in no case did *n* exceed a value of five.

The present work describes the separation of sheep liver folates on columns of DEAE-Sephadex (Pharmacia, Uppsala, Sweden) by elution with phosphate

* Abbreviations: PteGlu, pteroyl-L-glutamic acid; H₂PteGlu and H₄PteGlu, 7,8-dihydro- and 5,6,7,8-tetrahydro-pteroyl-L-glutamic acid; 5-CH₃-H₄PteGlu, 5-methyltetrahydropteroyl-L-glutamic acid; 5 (and 10)-HCO-H₄PteGlu, 5 (and 10)-formyltetrahydropteroyl-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.

buffer. Evidence is presented that sheep liver contains the several series of folates listed above with all values of n up to seven.

Materials and Methods

Animals

Liver folates were examined in nine Australian Merino ewes at 4 years of age on two different dietary regimes.

Four animals were removed from pasture and fed *ad libitum* for 7–80 days on a stock diet of equal parts by weight of wheaten hay-chaff and dried lucerne-chaff. Food intakes were 1000–1200 g/day. One of the animals received 53 μ Ci of $[2-^{14}\text{C}]\text{PteGlu}$ intravenously in ten equal doses over 5 days. It was killed 3 days after the last injection and the liver was used to determine the efficiency of extraction of liver folates. The extract contained most of the labelled folates in the liver and was used to determine conditions for optimum separation of folates by column chromatography.

The five remaining animals were fed on a diet containing (by weight) 94% wheaten hay-chaff, 5% washed wheat gluten and 1% NaCl. Each animal received 35 mg of Cu^{2+} (as CuSO_4 in water) per week by drench and 25 ml of cod liver oil per week (in two equal drenches). Vitamin B_{12} (250 μ g of cyanocobalamin in 0.5 ml of water) was given weekly to each animal by intramuscular injection. Food intakes were progressively restricted over 10–15 weeks from an initial intake of 1060 g/day to a final intake of 202 ± 27 (S.E.M.) g/day when the animals were killed. During this time body weights declined from 42.5 ± 0.8 (S.E.M.) kg to 32.9 ± 1.0 (S.E.M.) kg (Smith & Osborne-White, 1973).

All animals were killed by captive bolt pistol, then the throats were cut and the livers rapidly removed for preparation of liver extracts.

Extraction of liver folates

The method used was based on that of Bird *et al.* (1965). All extracts were made at a final concentration of 1% potassium ascorbate (w/v, calculated as anhydrous ascorbic acid) at pH 6.0. Livers were rapidly cut into pieces (1–2 g); the pieces (up to 300 g) were immediately chilled by agitation for 1 min in two successive lots of 15–20 vol. of 0.25 M-sucrose at 0°C. Lots (about 20 g) of chilled liver pieces were blotted dry, weighed and homogenized in 24 ml of 1.83% ascorbate for 45 s at top speed in a Servall Omnimixer (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) kept cold in an ice-water bath. Four 10 ml portions from each of four or five successive homogenates were heated for 10 min with intermittent stirring in a large water bath at 95°C then chilled rapidly in an ice-water bath. A further 10 ml of 1% ascorbate was added to each tube,

the contents were homogenized briefly and centrifuged for 15 min at 15000 g and 0°C. The supernatants were stored at –20°C; the folates present appeared to be stable for at least 6 months.

Efficiency of extraction was determined with the liver of the animal injected with $[2-^{14}\text{C}]\text{PteGlu}$. The liver was found to contain 19% of the injected ^{14}C . Initial extracts were prepared as described above and the residues were then re-extracted three times in volumes of 1% ascorbate equivalent to those removed in the previous centrifugal supernatants. Re-extractions were for 10 min at either 0° or 95°C with intermittent stirring and the final residue was resuspended in 1% ascorbate for estimation of ^{14}C and dry matter. The ^{14}C content of the residue and of all extracts was determined by liquid-scintillation counting [0.1 ml of extract or slurry in 5 ml of Bray's (1960) scintillator fluid with and without internal standard of $[\text{methyl-}^{14}\text{C}]\text{toluene}$].

Chromatography of liver folates

A uniform sample of DEAE-Sephadex A25 (particle size 40–120 μ m) was set aside and used to prepare all columns. A portion (30 g) of gel beads was allowed to swell overnight in 1 litre of water then elutriated five times in lots of 1.5 litres of water to remove fines. The sedimented gel was stirred with two consecutive lots of 250 ml of 0.5 M-KOH, followed by elutriation with six lots of 1.5–2.0 litres of water. Two consecutive treatments with 250 ml portions of 0.5 M-potassium phosphate buffer (pH 6.0) were then given followed by a further six washes with 2 litre lots of water.

Columns were packed under gravity and washed through with water from a peristaltic pump (0.68 ml/min) until the eluate contained <0.5 mM-potassium phosphate. Immediately before applying the sample the column was washed through with 50 ml of 0.2% ascorbate (pH 6.0). The final dimensions of the compacted column were 80 cm $\times$ 0.9 cm diameter. Columns were maintained at 22°C by a lagged jacket of circulating water delivered from a constant-temperature bath. The latter, together with the column and a fraction collector, was set up in a darkened cold-room at 0–2°C.

Liver extract in 1% ascorbate (equivalent to about 9 g of fresh liver) was reheated at 95°C for 10 min, chilled and centrifuged to remove residual protein. The supernatant was concentrated to about 5 ml by freeze-drying but without taking to dryness. The concentrated solution was applied to the column and washed in with three lots of 1 ml of 1% ascorbate (pH 6.0). The column was eluted with potassium phosphate buffer (pH 6.0), initially as a gradient but for the most part at a concentration of 1.0 M (Fig. 1). The mixing chamber contained 300 ml of 0.2% ascorbate (pH 6.0) and the reservoir contained 0.2%

ascorbate in 1.0M-potassium phosphate (pH6.0). The rate of elution (0.68ml/min) was controlled by a peristaltic pump. Fractions (9.8ml) of eluate were delivered by a fraction collector into 360 tubes each containing 0.2ml of 40% (w/v) ascorbate (pH6.0). Twice daily the newly eluted fractions were removed and stoppered tightly under N₂.

Determination of ¹⁴C (where appropriate) was carried out on samples from each tube but for microbiological assay consecutive groups of three (or sometimes nine) fractions were combined. The combined samples were kept under N₂ at -20°C.

Determination of ¹⁴C

Samples (0.7ml) of column eluate were added to 15.3ml portions of scintillator fluid, left in the dark for at least 8 h and counted for radioactivity for 10 min with a Packard Tri-Carb liquid-scintillation counter, model 3375 (Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.). The scintillator fluid was prepared by mixing 600ml of Triton X-100, 230ml of water and 700ml of toluene. The latter contained 2.8g of 2,5-diphenyloxazole and 70mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene.

The concentration of potassium phosphate in the samples affected both background (because of K⁺) and counting efficiency (which decreased with increasing P_i concentration). P_i concentrations were related to eluted volume as shown in Fig. 1 and these results, combined with a series of determinations of background counts at various concentrations of P_i in simulated eluate permitted interpolation of appropriate background values. Counting efficiency (counts as a fraction of ionizing events in the counting vial) was measured in each sample with an external standard source incorporated in the instrument. The values based on the external standard were corrected on the basis of an internal standardization of every twentieth sample with added [methyl-¹⁴C]toluene. Counting efficiency varied from 53.6% in absence of potassium phosphate to 23.5% when the eluate contained 1M-potassium phosphate. The calculations involved in measuring ¹⁴C were performed by computer. The computer was also programmed to estimate recovery of ¹⁴C applied to the column.

Microbiological assay

Three test organisms were used, *Lactobacillus casei* (A.T.C.C. 7469), *Pediococcus cerevisiae* (A.T.C.C. 8081) and *Streptococcus faecalis* R. (A.T.C.C. 8043). Media were purchased from Difco Laboratories Inc. (Detroit, Mich., U.S.A.), or, when these were unavailable, similar media were prepared in accordance with the formulations in the Difco manual (Ninth Edition, reprinted 1964). Bacto-Folic acid Casei medium was used for assay with *L. casei*,

Bacto-Folic T.E. medium was used for assay with *S. faecalis* and assays with *P. cerevisiae* were carried out with Bacto-C.F. medium adjusted to pH6.3 or pH5.3 with acetic acid.

All assays were performed by using the 'aseptic plus ascorbate' technique of Bakerman (1961) but in final volumes of 5ml containing 0.1% ascorbate. All samples and standards at whatever dilution and whether or not treated with conjugase (see below) were presented for assay in solutions containing 1% ascorbate, 0.2M-potassium phosphate and 0.1M-sodium acetate at pH6.0. Samples or standards (0.5ml) were added aseptically to 4.5ml of concentrated medium in Pyrex test tubes (150mm × 19mm external diam.). Tubes were inoculated and incubated at 37°C for 18h for *L. casei*, for 22h for *P. cerevisiae* and for 18h for *S. faecalis* before being chilled and the bacterial growth measured turbidimetrically at 660nm in 1cm-square cuvettes with a Shimadzu QV-50 spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan). The reference standard for all assays was (dl)-L-calcium leucovorin calculated as 1-L-5-HCO-H₄PteGlu. Assays were carried out in triplicate at each of two or three dilutions. The assay range for *P. cerevisiae* was restricted to 0–0.2ng/ml. (final concentrations).

Preparation and standardization of hog kidney conjugase

The preparation commonly used consists of a crude extract of pig kidney that has been allowed to autolyse before treating it with an anion-exchange resin to remove the folates that are released (Eigen & Shockman, 1963). For use with column eluates in the present work an additional treatment with Dowex-1 (Cl⁻ form) was given to decrease the folate content to <0.5ng/mg of protein. The preparation described by Eigen & Shockman (1963) (200ml at pH4.5) was passed through a column (26cm × 1.5cm diam.) of analytical grade Dowex-1 (X8, 200–400 mesh, Cl⁻ form). Eluate was collected in 10–20ml fractions and those fractions containing most of the protein were combined, readjusted to pH4.5 and stored at -20°C. Residual activity for *S. faecalis* was about the same as that for *L. casei* (0.2–0.4ng/mg of protein) but activity for *P. cerevisiae* was 30–40% lower. Preparations contained 12–18mg of protein/ml.

Before treatment with conjugase less than 5% of the folates extracted from sheep liver were available to *L. casei*. With each preparation of conjugase, tests were carried out to determine the minimum amount of enzyme necessary to release completely to *L. casei* a quantity of liver folate substantially in excess of the maximum likely to be encountered analytically. For most preparations about 1mg of conjugase protein incubated for 2h at 37°C and pH4.7 was sufficient to ensure complete release of 1ng of folate present as a

mixture of otherwise unavailable forms. Smaller amounts of protein led to smaller release but larger amounts caused no further net increase.

Treatment of samples with conjugase

Samples of column eluate (2.4 ml) were adjusted to pH 4.7 with 5M-acetic acid (glass electrode) and incubated with hog kidney conjugase (10–20 mg of protein) with shaking for 2 h at 37°C in 6 ml volumes containing 1% ascorbate, 0.4M-potassium phosphate and about 0.2M-sodium acetate at pH 4.7. After incubation samples were cooled, adjusted to pH 6.0 and made up to 12 ml containing final concentrations of 1% ascorbate, 0.2M-potassium phosphate and 0.1M-sodium acetate. Further dilutions for assay were made with buffer of the same composition.

Chemical determinations

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Phosphate was determined by the method of Sumner (1944).

Materials

Pteroylglutamic acid was obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Calcium leucovorin (Ca-dl-L-5-HCO-H₄PteGlu-5H₂O) was obtained from Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y., U.S.A. 10-HCO-H₄PteGlu was prepared from calcium leucovorin by the method of Rabinowitz (1963).

[2-¹⁴C]PteGlu and [methyl-¹⁴C]CH₃-H₄PteGlu were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [2-¹⁴C]H₄PteGlu was prepared by the method of Davis (1968) and [2-¹⁴C]-H₂PteGlu was prepared by the method of Blakley

(1960). Unlabelled H₄PteGlu for microbiological assay was prepared by the method of Rabinowitz & Pricer (1957).

2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from the Packard Instrument Co., Downers Grove, Ill., U.S.A.

Other materials were reagent-grade chemicals or the best commercially available grade. Glass-distilled water was used throughout.

Results

Extraction and chromatography of folates from the liver of sheep fed on a diet of wheaten hay-chaff and lucerne-chaff

Total folate activity in liver extracts. The folate activity in the livers of three sheep fed on the stock hay-chaff and lucerne-chaff diet are shown in Table 1. After treatment with conjugase, material active only for *L. casei* (presumably 5-CH₃-H₄PteGlu) constituted 71.6% of the total activity of about 12.5 µg of folate/g. Activity for *P. cerevisiae* was somewhat less than that for *S. faecalis*.

Injection of an animal (no. 363) over 5 days with an amount of [2-¹⁴C]PteGlu equivalent to about 10% of the total liver folate, did not lead to a significant increase in liver folate concentrations three days after the last dose, although at that time the ¹⁴C present in the liver was 40.3% of that estimated to remain in the body. (Urinary excretion of ¹⁴C was 27.8 µCi up to the time of death and faecal excretion was <0.1 µCi.)

Efficiency of extraction of [2-¹⁴C]folates from liver. Table 2 shows that a single extraction in 1% ascorbate (pH 6.0) for 10 min at 95°C released about 83% of the [2-¹⁴C]folates in an homogenate of the liver of animal no. 363. A further 4% of the label was recovered by repeated re-extraction of the centrifugal residue at

Table 1. *Folates in normal sheep liver*

Microbiologically active folates (means ± S.E.M.) in the livers of three ewes fed *ad libitum* on a stock diet of equal parts of wheaten hay-chaff and dried lucerne-chaff and of a further animal (no. 363) fed on the same diet and injected intravenously with 53 µCi (467 µg) of [2-¹⁴C]PteGlu in ten equal doses over 5 days and killed 3 days later. The liver of the latter animal had retained 19.1% of the injected ¹⁴C.

Assay organism ...	Folate concn. (µg/g of liver)					
	<i>L. casei</i>		<i>P. cerevisiae</i>		<i>S. faecalis</i>	
	Before conjugase	After conjugase	Before conjugase	After conjugase	Before conjugase	After conjugase
Animals not injected with ¹⁴ C folate	0.337 ± 0.075 (3)	12.52 ± 1.21 (3)	0.018 ± 0.003 (3)	3.13 ± 0.57 (3)	0.048 ± 0.004 (3)	3.55 ± 0.20 (3)
Animal no. 363 injected with ¹⁴ C folate	0.70	9.75	0.050	1.54	0.113	3.06

Table 2. Efficiency of extraction of [2-¹⁴C]folate from sheep liver

Four portions of an homogenate from the liver of animal no. 363 (Table 1) were extracted at 95°C for 10 min in 1% ascorbate (pH 6.0) as described in the text. The centrifugal residues were re-extracted in 1% ascorbate three times at either 95° or 0°C. Measurements were made of ¹⁴C in the extracts and in the final residues and the latter were used to determine residual liver solids. The table shows the means ± S.E.M. of duplicate estimates of ¹⁴C in the various fractions examined.

Combined second, third and fourth extracts				Pellet of residual solids				Total ^{14}C in 4.76 g of liver (A + B + C) (nCi)	Fraction of ^{14}C not released on repeated washing (%)	Fraction of ^{14}C released in a single extraction at 95°C (%)
First extract		^{14}C in combined supernatants (nCi) (B)		^{14}C in pellet (nCi) (C)	^{14}C estimated to be in fluid phase (nCi)	^{14}C bound to solids (nCi)				
Temp. (°C)	^{14}C in supernatant (nCi) (A)	^{14}C estimated to be in fluid phase (nCi)	Temp. (°C)							
95	59.8 ± 1.4	80.0 ± 1.4	95	25.6 ± 1.5	13.0 ± 2.0	0.52 ± 0.12	12.4 ± 1.9	98.4 ± 4.9	12.6	83.4
95	61.5 ± 0.2	82.9 ± 0.7	0	22.4 ± 0.9	13.1 ± 0.9	0.61 ± 0.08	12.4 ± 0.8	97.0 ± 1.4	12.8	

either 0° or 95°C, but the remainder appeared to be tightly bound to the residual solids.

Chromatography of [2-¹⁴C]folate in liver extract. A portion of the extract equivalent to 15.5 g of liver from animal no. 363 contained 265 nCi of ¹⁴C and was applied to a column of DEAE-Sephadex. Elution of the column and measurement of ¹⁴C in the eluate was carried out as described in the Materials and Methods section. Recovery of radioactivity was complete in 320 fractions and is illustrated in Fig. 1 which also shows the profile of the [P_i] gradient. Several partly resolved components were eluted in the first 104 fractions followed by four well-separated peaks. The latter peaks together accounted for 76.5% of the total ¹⁴C present.

Separation and identification of liver folates in animals fed on restricted amounts of a diet of wheaten hay-chaff and gluten

Chromatographic separation and recovery of micro-biologically active folates in liver extracts. Chromatography of liver extracts of animals maintained on this dietary regime resulted in a reproducible pattern of folate elution as shown by microbiological assay. This is indicated in Fig. 2 which shows the mean profile of activity for the livers of five animals with each assay organism determined both with (a) and without (b) conjugase treatment. Recovery of activity for each of the organisms was essentially complete in 320 fractions (Table 3).

Two general features of the separation may be noted. First, in accord with the specificity of the assay organisms (Stokstad & Koch, 1967) activity for *L. casei* always equalled or exceeded that for *S. faecalis*, which, in turn, equalled or exceeded that for *P. cerevisiae*. The extent of the differences between the microbiological responses was characteristic of elution position and constitutes evidence for the nature of the folates present. Secondly, very little of the activity past fraction 80 was available to the organisms until the material had been treated with conjugase, and even before fraction 80 there was generally some net microbiological response to conjugase treatment. By far the greater part of the folates in sheep liver seemed to be present in polyglutamate form and emerged from the column later than the mono- and di-glutamates.

Elution positions of authentic pteroylglutamates. These were determined individually and are shown by the arrows in Fig. 3. Little separation was obtained between 5-CH₃-H₄PteGlu and H₄PteGlu. Elution of PteGlu was only slightly later than that of H₂PteGlu which in turn followed closely after H₄PteGlu. The formyltetrahydrofolates were well separated from one another and from the other pteroylglutamates tested.

Chromatography of liver folates after treatment with conjugase. A combined extract from the livers of two

Fig. 1. Separation of liver folates from a sheep injected with $[2-^{14}\text{C}]\text{PteGlu}$

A ewe was fed on a stock diet of wheaten hay-chaff and dried lucerne-chaff and injected intravenously with $53\ \mu\text{Ci}$ ($1.06\ \mu\text{mol}$) of $[2-^{14}\text{C}]\text{PteGlu}$ in ten equal doses over 5 days. Three days after the last injection the animal was killed and the liver was found to contain $10.1\ \mu\text{Ci}$ of $[2-^{14}\text{C}]\text{folate}$. The folates extracted from 15.5 g of liver were adsorbed on DEAE-Sephadex and eluted with potassium phosphate (pH 6.0) in 360 fractions each of 9.8 ml, as described in the text. ----, Concentration of P_i in the eluate; —, radioactivity. Recovery of ^{14}C was complete in 320 fractions.

Fig. 2. Microbiological activity of chromatographically separated folates from the livers of sheep

The graph shows the mean results of microbiological assays of corresponding eluant fractions from chromatograms of liver extracts of five sheep. The animals were all eating restricted amounts (about 200 g/day) of a diet of wheaten hay-chaff and gluten and were injected weekly with vitamin B_{12} . Quantities of extract equivalent to about 9 g of liver were chromatographed on DEAE-Sephadex as described in the text. Fractions (10 ml) were combined in groups of three for assay. Assays were performed both after (a) and before (b) treatment of eluate with hog kidney conjugase. The assays shown in the Figure were with *L. casei* ($\circ$); *S. faecalis* (Δ); and *P. cerevisiae* (at pH 6.3) ($\bullet$). Recovery of activity for all three organisms was essentially complete in 320 fractions.

Table 3. *Folates in the livers of sheep fed on restricted amounts of food*

Folate activity for three assay organisms in the livers of five ewes fed on amounts of hay-chaff and gluten that were progressively restricted from 1060 g/day to about 200 g/day over 10–15 weeks. Means $\pm$ S.E.M. for five assays are shown both for direct assay of liver extracts before and after conjugase treatment and for total recovery after treatment with conjugase when the same extracts were eluted from DEAE-Sephadex as shown in Fig. 2(a).

Assay	Direct assay of liver extract		Recovery in 3200 ml of column effluent (after conjugase treatment) ($\mu\text{g/g}$ of liver)
	Before conjugase treatment ($\mu\text{g/g}$ of liver)	After conjugase treatment ($\mu\text{g/g}$ of liver)	
<i>L. casei</i>	0.364 ± 0.085	5.22 ± 0.91	5.09 ± 0.82
<i>P. cerevisiae</i> (pH 6.3)	0.045 ± 0.010	1.52 ± 0.45	1.57 ± 0.36
<i>S. faecalis</i>	0.109 ± 0.022	2.55 ± 0.73	2.32 ± 0.56

ewes on a similar dietary regime to those referred to in Fig. 2, was treated with an excess of conjugase before chromatography. The eluted fractions were assayed individually instead of in groups of three, by using all four assay procedures. The microbiological activities, corrected for activity contributed by the conjugase preparation are shown in Fig. 3 to occur in four distinct peaks.

Two of the peaks corresponded in elution positions to 10-HCO-H₄PteGlu (fraction 24) and 5-HCO-H₄PteGlu (fraction 44). These identities were confirmed by the equivalent activity of the eluted material in all four assays. The large peak around fraction 55 appeared to contain two components. Material active only for *L. casei* emerged around fraction 55 and was superimposed on a second peak with maximum activity at fraction 57. The latter material was equally active for *S. faecalis* and *P. cerevisiae* (pH 5.3) but had only 47% of that activity for *P. cerevisiae* (pH 6.3). The former peak corresponded both in elution position and microbiological activity to 5-CH₃-H₄PteGlu whereas the latter corresponded to H₄PteGlu.

Neither *P. cerevisiae* (Keresztesy & Donaldson, 1961) nor *S. faecalis* (Silverman *et al.*, 1961) responds to 5-CH₃-H₄PteGlu and the presence of activity for *P. cerevisiae* is diagnostic of fully reduced folates. As shown later a 50% response of *P. cerevisiae* (pH 6.3) relative to *P. cerevisiae* (pH 5.3) is characteristic of H₄PteGlu. The peak of activity around fraction 57 is therefore entirely consistent with the presence of H₄PteGlu. Neither H₂PteGlu nor PteGlu was present in significant quantity.

The small peak of activity around fraction 7 was not identified and is discussed below. Inspection of Fig. 3 shows that this minor peak contained activity both for *L. casei* and for *P. cerevisiae* (at pH 5.3 and 6.3). Apart from this activity and a further minor peak around fraction 33 all the folate recovered from the column could be accounted for in the four major components mentioned.

Differential assay of tetrahydrofolates, formyl-tetrahydrofolates and methyltetrahydrofolates. Tetrahydropteroylglutamate may be assayed with *P. cerevisiae* in the presence of ascorbate but the extent of the response relative to that for 5-HCO-H₄PteGlu depends both on the pH value of the medium and on the concentration of H₄PteGlu. At pH 6.3 concentrations of H₄PteGlu below 0.3 ng/ml cause a growth response in *P. cerevisiae* equal to that with about half the molar equivalent of 5-HCO-H₄PteGlu, but above 0.3 ng/ml the response approaches that for 5-HCO-H₄PteGlu (Bakerman, 1961). Donaldson & Keresztesy (1959) successfully assayed H₄PteGlu against a standard of 5-HCO-H₄PteGlu with *P. cerevisiae* (pH 6.3) by use of a correction factor of 2.3. They pointed out that no discontinuity in response to H₄PteGlu occurs with *P. cerevisiae* at pH 5.3 and Bird *et al.* (1969) showed that at pH 5.3 the response of *P. cerevisiae* to H₄PteGlu and 5-HCO-H₄PteGlu is equivalent at all concentrations. Fig. 4 shows that under our conditions of assay synthetic H₄PteGlu was 48% as active for *P. cerevisiae* (pH 6.3) as it was for *P. cerevisiae* (pH 5.3). From Fig. 3 no other chromatographically distinguishable monoglutamate exhibited this characteristic. It therefore becomes possible to distinguish quantitatively between H₄PteGlu and HCO-H₄PteGlu by the differential response of *P. cerevisiae* at pH 5.3 and pH 6.3 provided the assay range is kept below 0.3 ng/ml. Under these assay conditions:

$$\text{H}_4\text{PteGlu} = 2 [\textit{P. cerevisiae} \text{ (pH 5.3)} - \textit{P. cerevisiae} \text{ (pH 6.3)}] \text{ activity} \quad (1)$$

and

$$\text{HCO-H}_4\text{PteGlu} = [2 \textit{P. cerevisiae} \text{ (pH 6.3)} - \textit{P. cerevisiae} \text{ (pH 5.3)}] \text{ activity} \quad (2)$$

The relationship between activity for *P. cerevisiae* (pH 5.3) and that for *S. faecalis* among liver folates was established with eluates from chromatograms of

Fig. 3. Folates of sheep liver chromatographed after treatment with conjugase compared with authentic pteroylglutamates

Liver extracts were prepared from two sheep (nos. 007 and 024) on a similar dietary regime to those of Fig. 2 but at a food intake restricted to 319 g/day and 243 g/day, respectively. Folate activity for *L. casei* after conjugase treatment was equivalent to 3.83 µg/g of liver and 4.50 µg/g of liver respectively. A mixture containing 1.25 µg of folate from each of the extracts was incubated with conjugase (416 mg of protein containing 102 ng of folate activity for *L. casei*) for 2 h at 37°C and pH 4.7. The figure shows activity for *L. casei* (○), *S. faecalis* (△), *P. cerevisiae* (pH 6.3) (●) and *P. cerevisiae* (pH 5.3) (▲) on chromatographing the mixture. The results have been corrected for the contribution of the conjugase preparation (chromatographed and assayed separately). Recovery of activity was essentially complete in 70 fractions. The arrows show the elution positions of 1 µg quantities of authentic pteroylglutamates procured or prepared as described in the Materials and Methods section. 10-HCO-H₄PteGlu (1) and 5-HCO-H₄PteGlu (2) were detected by microbiological assay with *L. casei*. 5-CH₃-H₄PteGlu (3), H₄PteGlu (4), H₂PteGlu (5) and PteGlu (6) were labelled with ¹⁴C and were detected by radioactivity. H₄PteGlu was further identified by microbiological assay with *P. cerevisiae* (pH 5.3) and H₂PteGlu and PteGlu were further identified by their activity for *L. casei*. [Methyl-¹⁴C]CH₃-H₄PteGlu was co-chromatographed with one of the liver extracts but the other standards were chromatographed alone.

two further extracts of the livers of sheep on a similar dietary regime to that appropriate to Fig. 2.

Eluted fractions were combined in groups of three or nine for assay. In only one region (fractions 50–70) of the elution profile for one of the extracts was activity for *S. faecalis* clearly greater than that for *P. cerevisiae* (pH 5.3). After excluding the results for that part of the profile in both chromatograms, a statistical comparison of the two assays was made for all samples containing more than 1 ng of folate/ml active for *S. faecalis*. Mean activity for *P. cerevisiae* (pH 5.3) was 3.380 ng/ml whereas that for *S. faecalis* was 3.348 ng/ml (59 observations). The mean difference (0.032 ng/ml) had a standard error of 0.121 and was not significantly different from zero (*t* test). Thus except in a restricted range of the elution profile of some samples (where H₂PteGlu or PteGlu would have emerged) activity for *S. faecalis* and *P. cerevisiae* (pH 5.3) was equivalent and the folates leading to the *S. faecalis* response were fully reduced (see Fig. 3). With the restriction noted it therefore becomes possible to substitute *S. faecalis* activity for that of

P. cerevisiae activity (pH 5.3) in equations (1) and (2) to give equations (3) and (4).

$$\text{H}_4\text{PteGlu} = 2 [\textit{S. faecalis} \\ - \textit{P. cerevisiae} (\text{pH } 6.3)] \text{ activity} \quad (3)$$

and

$$\text{HCO-H}_4\text{PteGlu} = [2 \textit{P. cerevisiae} (\text{pH } 6.3) \\ - \textit{S. faecalis}] \text{ activity} \quad (4)$$

Inspection of Fig. 2 shows that the ratio *S. faecalis*/*P. cerevisiae* (pH 6.3) activity varied characteristically as elution proceeded and reached a maximum of about 2 around fraction 56 and again between fractions 270 and 320. The 90 samples represented in the latter peak had a mean activity for *P. cerevisiae* (pH 6.3) of 1.83 ng/ml and for *S. faecalis* of 4.08 ng/ml. This ratio suggested that the final peak of activity for these two organisms consisted mainly of tetrahydro-folate, and led to further examination in other chromatograms. Four further extracts of sheep liver were chromatographed and the final part of the profile

was established with the *L. casei* assay. Comparison was then made between activity for *P. cerevisiae* (pH 6.3) and that for both *P. cerevisiae* (pH 5.3) and *S. faecalis* in that part of the profile corresponding to fractions 270–320 in Fig. 2. The fractions were combined into 28 samples each of 90 ml for assay, and the results were expressed as regressions of *P. cerevisiae* (pH 5.3) or *S. faecalis* against *P. cerevisiae* (pH 6.3). Both regressions were linear with intercepts not significantly different from zero and they did not differ significantly from one another (*t* test). The two regressions were therefore combined. The mean activity between *P. cerevisiae* (pH 5.3) and *S. faecalis* regressed on *P. cerevisiae* (pH 6.3) showed an intercept not significantly different from zero and a slope of 1.925 with s.e. 0.079. Thus the final peak of activity for *S. faecalis* consisted essentially of fully reduced folate in which the ratio *P. cerevisiae* (pH 5.3)/*P. cerevisiae* (pH 6.3) activity was characteristic of tetrahydrofolate.

The difference between activity for *L. casei* and that for *S. faecalis* of the folates in mammalian tissues is generally accepted as being due to 5-CH₃-H₄PteGlu. Pteric acid and its formylated derivatives are active for *S. faecalis* and not for *L. casei* (Usdin, 1959) but the equivalence of the activities for *S. faecalis* and *P. cerevisiae* (pH 5.3) found in the present work (except where H₂PteGlu or PteGlu emerge) indicates that pterates were not present in significant amounts and permits the use of eqn. (5).

$$5\text{-CH}_3\text{-H}_4\text{PteGlu} = (\textit{L. casei} - \textit{S. faecalis}) \text{ activity (5)}$$

Methyltetrahydrofolates and microbiologically similar substances in sheep liver. The application of eqn. (5) to the profiles shown in Fig. 2 is given in Fig. 5. At least nine peaks may be readily distinguished, that at fraction 55 corresponding to the elution position of 5-CH₃-H₄-PteGlu. The regularity of this profile

Fig. 4. Activity of tetrahydrofolate for *P. cerevisiae*

A sample of tetrahydrofolate was prepared by the method of Rabinowitz & Pricer (1957) and assayed by *P. cerevisiae* at pH 5.3 and 6.3. The figure shows the relationship between the two assays. Each point represents the mean of triplicate assays for each method.

Fig. 5. Folates in sheep liver active only for *L. casei*

The values are derived from Fig. 2(a) and express the amount by which activity for *L. casei* exceeded that for *S. faecalis*. Standard 5-CH₃-H₄PteGlu was eluted at fraction 55 (arrow) and is defined in the inset as peak no. 1. The point of maximum concentration in the eluate of subsequent components was related as shown in the inset to the logarithm of the quantity of P₁ that had traversed the column to that point.

Table 4. Availability of successively eluted peaks of (*L. casei*–*S. faecalis*) activity without treatment with conjugase

Defining authentic 5-CH₃-H₄PteGlu eluted around fraction 56 as peak no. 1 with 100% availability to *L. casei*, the successive net *L. casei* peaks seen in Fig. 5 were progressively less available to the organism without conjugase treatment (cf. Fig. 2*b*). In the table the (*L. casei*–*S. faecalis*) activity of appropriate sequences of fractions in Fig. 2(*b*) (without conjugase treatment) are expressed as percentages of the activities in peaks 2–7 in Fig. 5.

5-CH ₃ -H ₄ PteGlu (peak no.)	Maximum activity at tube no.	Availability to <i>L. casei</i> before conjugase treatment (%)
1	56	100
2	70	91
3	90	39
4	116	17
5	155	4
6	212	1
7	294	0

suggested a relationship between the emergence of successive peaks and buffer composition, but from Fig. 1 the buffer composition was constant from about fraction 100. On examination the unexpected relationship shown in the inset to Fig. 5 emerged. Defining authentic 5-CH₃-H₄PteGlu as peak M1 successive peaks were eluted at positions related linearly to the logarithm of the quantity of P₁ that had traversed the column.

Preceding the seven net *L. casei* peaks represented by this relationship were two other peaks with maxima at fractions 23 and 38 (referred to as peaks X2 and X3 respectively). Comparison of Figs. 2(*a*) and 2(*b*) shows that much of the activity responsible for those peaks appeared only after treatment with conjugase. A response to conjugase is also apparent in the (*L. casei*–*S. faecalis*) activity differential around fraction 56. The latter response cannot have been due to 5-CH₃-H₄PteGlu and suggests the presence at this position of a further conjugated component (designated peak X4) in addition to 5-CH₃-H₄PteGlu.

The nature of the active material in peaks X2, X3 and X4 is unknown but the peaks may represent polyglutamate forms of the unidentified material active for *L. casei* that was eluted around fraction 7 when conjugase-treated extract was chromatographed (Fig. 3). The latter component was present in small amounts in most extracts chromatographed and is designated peak X1.

The decreasing availability of successive peaks of the methyltetrahydrofolate series to *L. casei* without prior conjugase treatment is shown in Table 4. Although the second peak appeared to be almost fully available to *L. casei* subsequent peaks gave progressively diminishing responses.

Tetrahydrofolates in sheep liver. The profile of the differential assay for H₄PteGlu [eqn. (3)] derived from the data of Fig. 2(*a*) is shown in Fig. 6. Seven well-separated peaks were present, preceded by a shoulder around fraction 40. Defining as peak H1 the material corresponding to authentic H₄PteGlu that eluted around fraction 58, the inset shows a relationship between the position of successive peaks and the logarithm of eluant [P₁] similar to that found for methyltetrahydrofolates. Comparison of Fig. 6 with Fig. 5 shows a small but perceptible separation between corresponding peaks of tetrahydrofolate and methyltetrahydrofolate.

The nature of the shoulder around fraction 40 is unknown.

Formyltetrahydrofolates in sheep liver. The residual activity for *L. casei* that was not accounted for either as methyltetrahydrofolate (with peaks X2, X3 and X4) or as tetrahydrofolate, is expressed by eqn. (4) and may be attributed to the presence of 5-HCO-H₄PteGlu_n and 10-HCO-H₄PteGlu_n. The two classes of formyltetrahydrofolates cannot be distinguished by microbiological assays carried out in the presence of ascorbate (Bird *et al.*, 1969).

Fig. 7 shows the values of eqn. (4) derived from the data of Fig. 2(*a*). Although the quantities of many of the components were quite small, ten peaks may be distinguished with maxima at fractions 24, 32, 44, 59, 65, 80, 99, 125, 170 and 233. Most of these differential peaks were perceptible in most of the individual chromatograms and in addition an eleventh differential peak around fraction 53 could be distinguished in some chromatograms. (In Fig. 7 the latter component appears only as an asymmetry of the previous peak.) The eleven differential peaks are referred to in their order of elution as HCO-H₄PteGlu peaks F1–F11.

Fig. 6. Folates in sheep liver more active for *S. faecalis* than for *P. cerevisiae* (pH 6.3)

The data were taken from Fig. 2(a) and express the quantity $2 [S. faecalis - P. cerevisiae \text{ (pH 6.3)}]$ activity. Standard $H_4PteGlu$, when chromatographed alone, eluted around fraction 56 (arrow) and is defined as peak no. 1. Elution of subsequent components was related to the logarithm of the quantity of eluant P_1 as shown in the inset.

Fig. 7. Residual folates in sheep liver

The graph shows the remaining activity for *L. casei* in Fig. 2(a) that was not accounted for in Figs. 5 and 6. The values were obtained from the assay differential $[2 P. cerevisiae \text{ (pH 6.3)} - S. faecalis]$ activity and represent an expression of the activity of formyltetrahydrofolate. The arrows marked A and B show the elution positions of authentic $10\text{-HCO-H}_4PteGlu$ and $5\text{-HCO-H}_4PteGlu$ respectively. The inset offers a plausible partial solution to the identities of the members of two overlapping series of folates each of seven components of which $10\text{-HCO-H}_4PteGlu$ and $5\text{-HCO-H}_4PteGlu$ were the first components.

Some distinctions between 5-formyl and 10-formyl components may be made. First, the two differential peaks that emerge at fractions 24 and 32 precede the appearance of $5\text{-HCO-H}_4PteGlu$. Authentic $10\text{-HCO-H}_4PteGlu$ corresponds to the first position and the peak at fraction 32 may be taken as the second component in a 10-formyltetrahydrofolate series. If the elution of formyltetrahydrofolates is related to the logarithm of eluted $[P_1]$ in a similar way to that of the two series already discussed, then the first two peaks should define the slope of the line. Secondly, from Fig. 3 and another similar chromatogram, the amounts

of $10\text{-HCO-H}_4PteGlu$ and $5\text{-HCO-H}_4PteGlu$ present in the extracts were comparable and in considering the possible distribution of the two forms constituting the profile of Fig. 7 due attention has been paid to this fact.

The inset to Fig. 7 and Table 5 suggest a plausible partial solution to the identities of the eleven substances detected that is consistent with the restrictions noted. This is not a unique solution. The assumption that each of the two formyltetrahydrofolate series contained seven members like the other two series detected may not be justified.

Table 5. *Proposed identities of the folates leading to the microbiological responses shown in Fig. 2*

The evidence relating to the identity of the various components is given in the text.

Differential assay	Peak no.	Maximum activity at fraction no.	Proposed identity
<i>(L. casei - S. faecalis)</i> activity	X1	7	Unknown
	X2	23	Unknown polyglutamates possibly converted into peak X1 on treatment with conjugase
	X3	38	
	X4	56	
	M1	56	5-CH ₃ -H ₄ PteGlu
	M2	70	5-CH ₃ -H ₄ PteGlu ₂
	M3	90	5-CH ₃ -H ₄ PteGlu ₃
	M4	116	5-CH ₃ -H ₄ PteGlu ₄
	M5	155	5-CH ₃ -H ₄ PteGlu ₅
	M6	212	5-CH ₃ -H ₄ PteGlu ₆
	M7	294	5-CH ₃ -H ₄ PteGlu ₇
2 [<i>S. faecalis</i> - <i>P. cerevisiae</i> (pH6.3)] activity	H1	59	H ₄ PteGlu
	H2	71	H ₄ PteGlu ₂
	H3	93	H ₄ PteGlu ₃
	H4	123	H ₄ PteGlu ₄
	H5	158	H ₄ PteGlu ₅
	H6	218	H ₄ PteGlu ₆
	H7	297	H ₄ PteGlu ₇
[2 <i>P. cerevisiae</i> (pH6.3) - <i>S. faecalis</i>] activity	F1	24	10-HCO-H ₄ PteGlu
	F2	32	10-HCO-H ₄ PteGlu ₂
	F3	44	10-HCO-H ₄ PteGlu ₃ + 5-HCO-H ₄ PteGlu
	F4	53	5-HCO-H ₄ PteGlu ₂
	F5	59	10-HCO-H ₄ PteGlu ₄
	F6	65	5-HCO-H ₄ PteGlu ₃
	F7	80	10-HCO-H ₄ PteGlu ₅
	F8	99	5-HCO-H ₄ PteGlu ₄
	F9	125	10-HCO-H ₄ PteGlu ₆ + 5-HCO-H ₄ PteGlu ₅
	F10	170	10-HCO-H ₄ PteGlu ₇ + 5-HCO-H ₄ PteGlu ₆
	F11	233	5-HCO-H ₄ PteGlu ₇

Discussion

Mammalian tissue folates that are active only for *L. casei* are generally assumed to be methyltetrahydrofolates, and the present finding of at least three peaks of activity of this kind chromatographically distinguishable from the methyltetrahydrofolate series may indicate the presence in liver of a hitherto unrecognized form of folate. This is consistent with the appearance of additional *L. casei* activity around fraction 7 when conjugase treatment preceded chromatography, although the latter material also showed activity for *P. cerevisiae*. The relatively early elution of this material both with and without prior conjugase treatment suggests a low anionic charge but both the presence of activity for *L. casei* and the fact that there was a response to conjugase indicates that the substances were pteroylglutamates. Unlike the sequence of peaks beginning with 5-CH₃-H₄PteGlu

there was no coherent relationship between elution positions of X1, X2, X3 and X4 and the quantity of P_i through the column.

The series of seven net *L. casei* peaks beginning with 5-CH₃-H₄PteGlu very probably consists of the mono- to hepta-glutamates of methyltetrahydropterolate. The diminishing availability to *L. casei* of successive peaks before conjugase (γ -glutamylcarboxypeptidase) treatment indicates both the nature of the difference between the peaks and the sense in which the series of γ -glutamyl peptides emerged. The relationship found between the elution positions of successive peaks and the logarithm of eluant [P_i] is consistent with the progressive increase of anionic charge on increasing the number of glutamate residues in the chain and strongly supports the view that a common difference existed between successive peaks. From the known specificity of hog kidney conjugase (see

Blakley, 1969, pp. 30–32) this difference must be attributed to one or more γ -glutamyl peptide residues. The simplest assumption is a difference of a single glutamate residue between each successive peak and this is more readily reconciled with the availability of the methylfolates to *L. casei* (Table 4) than an assumption that the common difference was two or three glutamate residues (Stokstad & Koch, 1967). Shin *et al.* (1972) concluded that rat liver contained no pteroylpolyglutamate with more than five γ -glutamyl residues, and the pteroylheptaglutamate in yeast (Pfiffner *et al.*, 1946) is the longest chain reported for a natural folate.

The evidence for the presence of a series of tetrahydrofolates rests on the incomplete response to tetrahydrofolate of *P. cerevisiae* at pH 6.3 compared with the response at pH 5.3. From Fig. 3 this difference can be attributed only to the presence of H_4 PteGlu and the proportionality factor used of 50% activity of H_4 PteGlu for *P. cerevisiae* (pH 6.3) accords both with published results and with our own evidence.

The profiles for H_4 PteGlu (Fig. 6) and 5- CH_3 - H_4 PteGlu (Fig. 5) were generally similar and this may suggest that the differential assay of the former arose from some unsuspected microbiological activity of the latter. However, not only was there a perceptible displacement between the two profiles (see also Fig. 3) but under certain dietary conditions (vitamin B_{12} deficiency) peaks of 5- CH_3 - H_4 PteGlu are present that are not accompanied by proportional activity in the other two assays (Smith & Osborne-White, 1973). For reasons similar to those outlined for the methyltetrahydrofolates, the seven H_4 PteGlu peaks are taken to represent the mono- to hepta-glutamates.

The evidence relating to the identity of the formyltetrahydrofolates is less satisfactory, because of our inability to distinguish microbiologically between 10-HCO- H_4 PteGlu and 5-HCO- H_4 PteGlu. The presence of at least eleven components in the two classes of formyltetrahydrofolates and the high probability of some coincident elution, however, is readily consistent with the existence of two series each of seven members, as in the other major classes of folates.

In only a few extracts of normal sheep-liver has evidence for the presence of incompletely reduced folates been obtained, and then only in the region characteristic of H_2 PteGlu or PteGlu. The evidence consisted of activity for *S. faecalis* consistently in excess of that for *P. cerevisiae* (pH 5.3), or, in the absence of the latter assay, in excess of twice the activity for *P. cerevisiae* (pH 6.3). Unsubstituted folate or dihydrofolate seemed to be present only in very small amounts and only in the monoglutamate form. There was no evidence that polyglutamate forms of incompletely reduced folates of any kind occurred in liver.

Baugh & Krumdieck (1971) have suggested that the complexity of the natural folates is beyond the capacity of the present kind of analytical procedure to

resolve. They suggest further that crude preparations of conjugase are unsuitable analytical agents, both because of the presence of inhibitors of conjugase in the tissue extracts being analysed, and because of the possibility that preparations may contain other enzymes that interconvert folates. The present work shows that a meaningful resolution of tissue folates into almost thirty distinguishable forms was quite readily obtained. The adequacy of the conjugase preparation is indicated both by the complete conversion of all the folates in liver extracts into identifiable monoglutamates (Fig. 3) and by the equivalence of recoveries of total folates before and after chromatography (Table 3). The possibility of interconversion of folates during conjugase treatment remains but with adequate protection against oxidation is probably confined to non-enzymic conversion of 10-HCO- H_4 PteGlu into 5-HCO- H_4 PteGlu if it occurs at all (Bird *et al.*, 1969). Under the present conditions of assay the occurrence of this isomerization would be significant only in relation to the kind of results obtained in Fig. 3.

The general similarity of the profile of radioactive folates in Fig. 1 with the profile of *L. casei* activity of liver folates from animals on a different dietary regime in Fig. 3 suggests that the distribution of polyglutamate forms found in the latter may occur generally in sheep liver.

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