IDENTIFICATION OF FOLIC ACID COMPOUNDS IN RAT LIVER

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Summary. Separation of folates in rat liver has been investigated with DEAE-cellulose, Sephadex G-15 and G-25 columns. Liver extracts containing ^{3}H-labeled folates were obtained from rats injected intravenously with ^{3}H-folic acid (PteGlu) 24 hrs. before sacrifice. Livers were extracted with boiling ascorbate (pH 6.0). Co-chromatography with synthetic ^{14}C-pteroylglutamates indicates that 85-90% of liver folates are reduced forms of pteroylpentaglutamate ($H_4PteGlu_5$). 10-CHO-$H_4PteGlu_5$ was identified by co-chromatography of liver extracts with synthetic 10-CHO-$H_4PteGlu_5$. Monoglutamates represent 5% of total liver folates. The rest are di- or triglutamates.

INTRODUCTION

Since the discovery of heptaglutamates in yeast by Pfiffner et al. (1), many authors have reported the existence of polyglutamates in plants (2), algae (3), bacteria (4,5) and animal tissues (6,7). Very recently, Whitehead (8) reported that polyglutamate forms are the major portion of liver folates in rat, mouse and humans. However, more specific identification of the number of glutamyl residues in folate polyglutamates has been limited up to this time due to lack of specific analytical methods and unavailability of synthetic polyglutamates as reference standards.

In this paper we report separation and identification of natural folate derivatives by the combined techniques of gel filtration on Sephadex G-15 and application of ^{14}C-polyglutamate standards synthesized by the solid phase technique of Baugh and coworkers (9,10).

MATERIALS AND METHODS

Radioactive polyglutamyl derivatives of pteroic acid ($PteGlu_n$-U-^{14}C-Glu) were prepared and purified as described by Baugh et al. (9,10). 10-CHO-

$H_4PteGlu_5$ was synthesized according to Huennekens et al. (11).

Folic acid (Amersham/Searle Corp.) labeled with tritium on the 3',5' positions of the para-aminobenzoyl ring was purified by DEAE-cellulose column chromatography (12) one day before injection into rats. Male rats (Sprague-Dawley, 375-385 g) fed a nutritionally complete diet (13) were injected (femoral vein) with 20 μCi of a tritiated PteGlu solution (30 Ci/mM) and sacrificed 24 hrs. after injection. The liver was flushed with ice cold 0.2% sodium chloride to remove blood. Liver folates were extracted by dropping slices of liver tissue into 5 volumes of boiling 1.1% ascorbate solution (pH 6.0) to minimize autolysis of conjugates (Bird et al. (14)).

For DEAE-cellulose column chromatography (15), samples were eluted with a gradient of 50 ml 0.01 M potassium phosphate buffer (pH 6.0 with 0.2 M 2-mercaptoethanol) and 0.5 M phosphate buffer (pH 6.0 with 0.2 M mercaptoethanol). A 0.9 x 30 cm column was used, and one hundred 2.5 ml fractions were collected.

Sephadex G-15 and G-25 chromatography procedures were developed in our laboratory (16). Sephadex G-15, G-25 (fine) and G-25 (medium) were purchased from Pharmacia Fine Chemicals, Inc. A 0.75 x 200 cm glass column was packed with gel, the elution carried out with 0.025 potassium phosphate buffer (pH 7 with 0.2 M mercaptoethanol), and the eluate collected in 1.7 ml fractions. The 0.75 x 400 cm column was prepared by connecting two 0.75 x 200 cm glass columns with a pump.

The microbiological assay procedures used for all samples were essentially those described by Waters and Mollin (17) and later modified (18). Three organisms, L. casei, S. faecalis and P. cerevisiae, were used to identify the folate peaks obtained by column chromatography before and after being treated with hog kidney conjugase (19).

RESULTS AND DISCUSSION

Figs. 1 and 2 show the separation of folate derivatives from liver

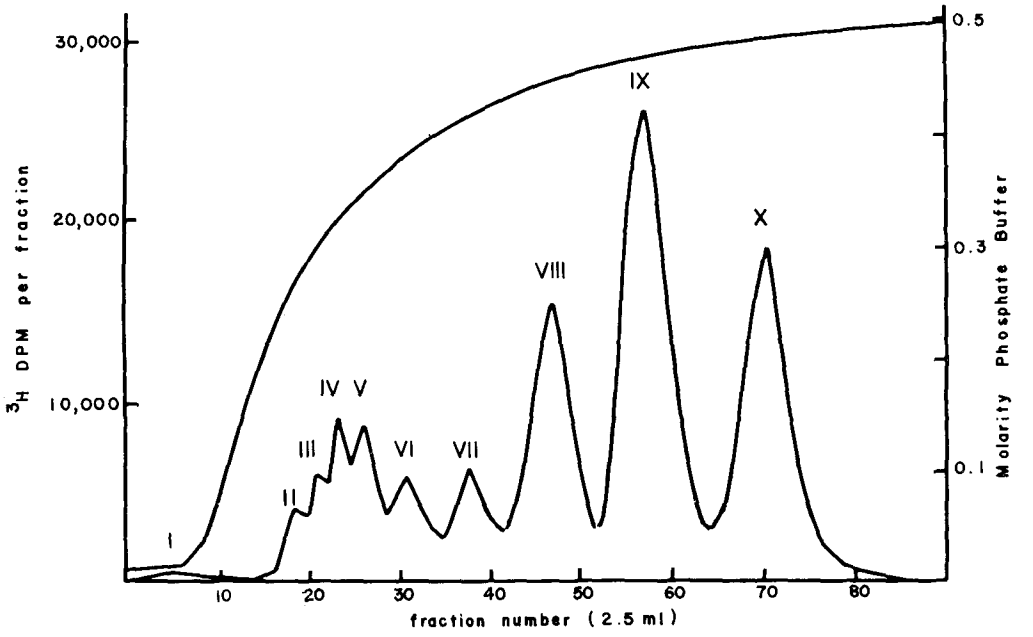

Fig. 1. DEAE-cellulose column chromatography of liver folates. The phosphate concentration gradient is superimposed on on the eluate patterns obtained from chromatography of an equivlent of 1 g fresh liver.

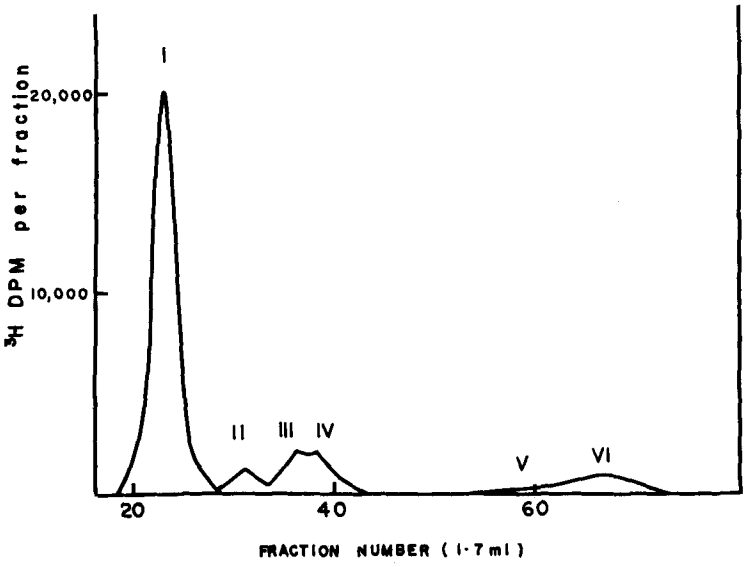

Fig. 2. Sephadex G-15 column chromatography of liver folates. Sample volume, 2.0 ml(0.4 g fresh liver); bed dimensions, 0.75 x 200 cm; void volume, fraction number 20; flow rate, 7.0 ml per hour; eluant, 0.025 M phosphate buffer, pH 7, with 0.2 M mercaptoethanol.

extracts on DEAE-cellulose and Sephadex G-15 columns. Table 1 shows elution patterns of standard folate compounds from a G-15 (0.75 x 200 cm) column.

Some of the DEAE peaks when rechromatographed on a Sephadex G-15 column proved to be combinations of two or more compounds. DEAE peaks III-VI, which were known to be largely reduced folate monoglutamates (15) were found to be mixed with folate di- and triglutamates (Table 1). DEAE peaks VII-X, presumably the polyglutamates, gave peak I on the G-15 column. This peak contained the polyglutamate forms (Table 1). Peak I from the G-15 column, when rechromatographed on DEAE-cellulose, gave 4 peaks corresponding to VII, VIII, IX and X. These results indicate that DEAE peaks VII-X are

Table 1. Elution Pattern of Standard Folates from the Sephadex G-15 Column (0.75 x 200 cm)

Standard Compounds	Molecular Weight	Fraction #	Elution Volume	Methods of Identification*
$PteGlu_7$	1,215	21	35.7	1,2,4
$PteGlu_6$	1,086	22	37.4	2,4
$PteGlu_5$	957	23	39.1	2,4
$10\text{-}CHO\text{-}H_4PteGlu_5$	989	22	37.4	2,4
$PteGlu_4$	828	25	42.5	2,4
$PteGlu_3$	700	34	57.8	2,4
$5\text{-}CH_3\text{-}H_4PteGlu_3$	718	32	54.4	2,4
$PteGlu_2$	571	38	64.6	2,4
$10\text{-}CHO\text{-}H_4PteGlu$	473	40	68.0	4
$5\text{-}CHO\text{-}H_4PteGlu$	473	54	91.8	4
$5\text{-}CH_3\text{-}H_4PteGlu$	459	72	122.4	2,4
$H_4PteGlu$	445	72	122.4	3
PteGlu	441	72	122.4	1,2,4

*1) Microbiological assay 2) Radioactivity measurement 3) Figlu enzymatic assay (20) 4) Optical density measurement at 282 mμ (ε_{max}= 27.6 x 10^3, pH 7)

folate polyglutamates with more than 3 glutamate residues. The major part of tritium activity and microbiological activity with L. casei were in this polyglutamate portion.

To establish that the polyglutamates were contained in G-15 peak I (Fig.2), aliquots of peak I were applied to a long G-15 column (0.75 x 400 cm), together with synthetic $PteGlu_5$, $PteGlu_6$ and $PteGlu_7$ containing ^{14}C-glutamic acid (Fig. 3). The elution pattern in Fig. 3 shows that the molecular weights of liver polyglutamate forms applied were larger than pentaglutamate ($PteGlu_5$)

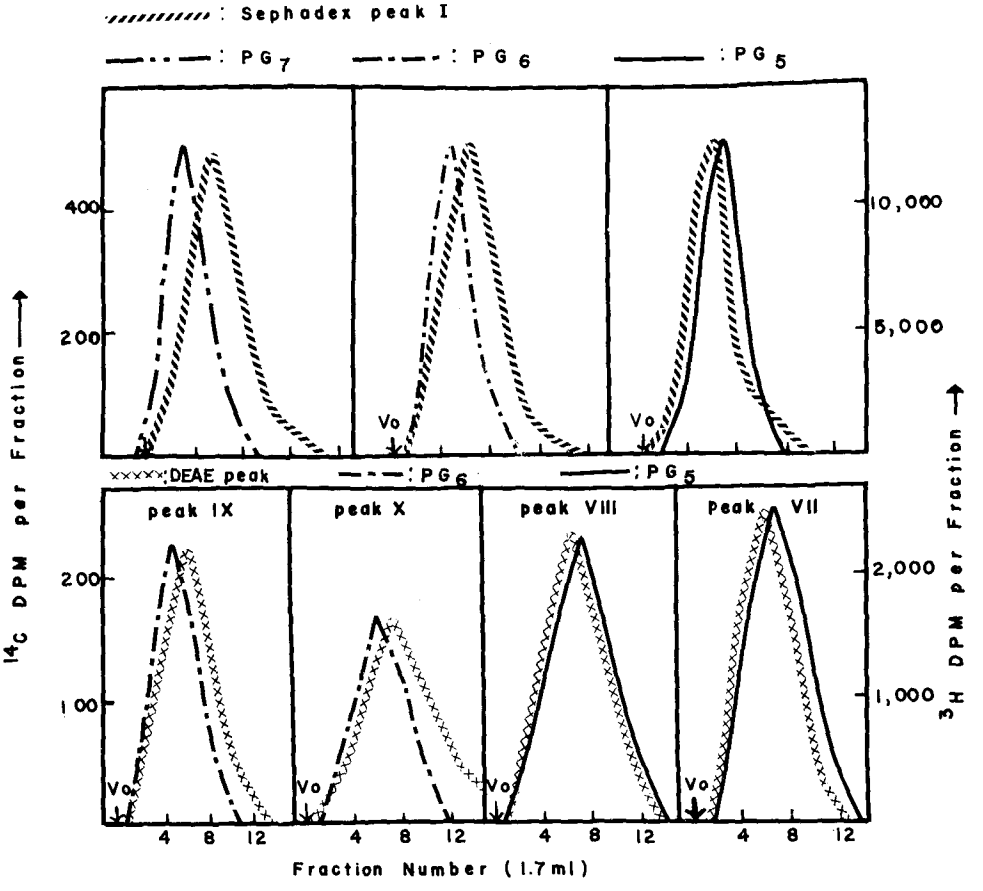

Fig. 3. Co-chromatography of Sephadex G-15 peak I and DEAE peaks VII-X with synthetic PG_5, PG_6, and PG_7(C-14) on Sephadex G-15 column. Sample volume, 2.0 ml; bed dimensions, 0.75 x 400 cm; flow rate, 7.0 ml/h; eluant, 0.025 M phosphate buffer, pH 7, with 0.2 M mercaptoethanol.

and smaller than hexaglutamate ($PteGlu_6$). Co-chromatography of DEAE peaks VII-X with synthetic polyglutamates on G-15 columns (0.75 x 400 cm) showed also the same results (Fig. 3).

For the further identification of these polyglutamates, we synthesized 10-CHO-$H_4PteGlu_5$ (11). The results of co-chromatography of liver polyglutamates with ^{14}C-10-CHO-$H_4PteGlu_5$ on G-15 and G-25 columns are shown in Fig. 4. These results establish that DEAE peak VII is 10-CHO-$H_4PteGlu_5$.

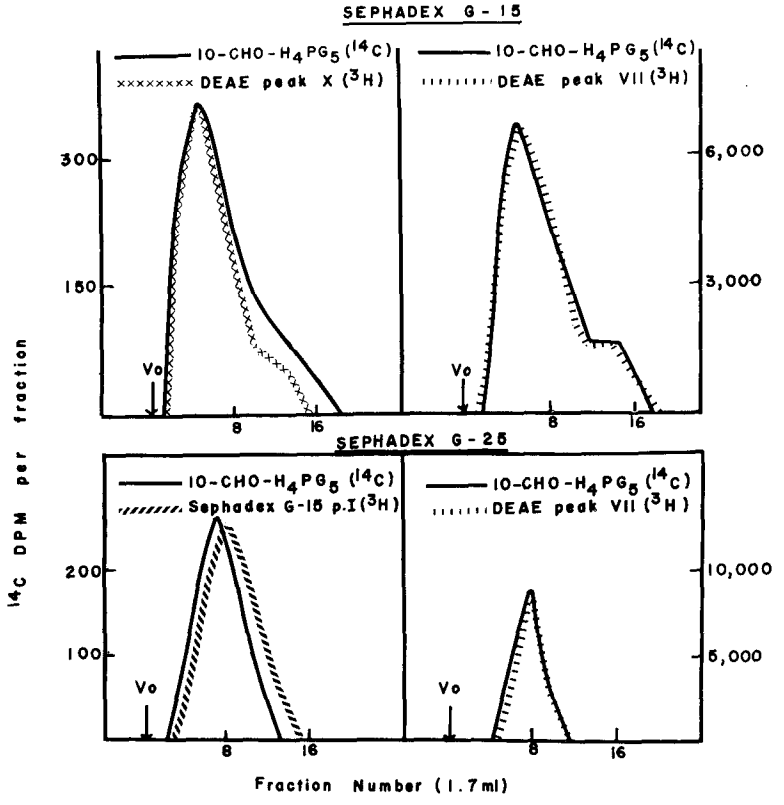

Fig. 4. Co-chromatography of Sephadex G-15 peak I and DEAE peaks with synthetic 10-CHO-H_4 PG_5 on Sephadex G-15 and G-25 columns. Sample volume, 2.0 ml; bed dimensions, 0.75 x 400 cm (G-15) and 0.75 x 200 cm(G-25); flow rate, 7.0 ml/h(G-15) and 15.0 ml/h(G-25); eluant, 0.025 M phosphate buffer, pH 7, with 0.2 M mercaptoethanol.

Identification of DEAE peaks VIII-X was done as follows (Tables 2 and 3). Peak VIII appears to be 5-CHO-$H_4PteGlu_5$ since it is eluted immediately

Table 2. Identification of Folate Derivatives in Peak Fractions Obtained by DEAE-Cellulose Column Chromatography of Liver Extracts

Peak	Folate Derivatives	Identification Methods*
I	Unidentified folate	
II	products	
III	10-CHO-H_4PteGlu	1,2
IV	5-CHO-H_4PteGlu	1,2
	10-CHO-H_4PteGlu$_2$	3
V	5-CH_3-H_4PteGlu	1,2
	5-CHO-H_4PteGlu$_2$	3
	10-CHO-H_4PteGlu$_3$	3
VI	H_4PteGlu	1,2
	5-CH_3PteGlu$_2$	3
	5-CHO-H_4PteGlu$_3$	3
VII	10-CHO-H_4PteGlu$_5$	2,3
	5-CH_3-H_4PteGlu$_3$	1,2
	H_4PteGlu$_2$	2
VIII	5-CHO-H_4PteGlu$_5$	3
IX	5-CH_3H_4PteGlu$_5$	3
X	H_4PteGlu$_5$	3

*1) An elution pattern of standard compound on DEAE-cellulose.
2) An elution pattern of standard compound on Sephadex G-15, G-25.
3) An elution pattern on Sephadex G-15.

after 10-CHO-H_4PteGlu$_5$ and responds to all 3 microorganisms. Peak IX appears to be chiefly 5-CH_3H_4PteGlu$_5$ according to differential microorganism response and by comparison with the elution order of reduced folate monoglutamate forms. A little growth was obtained with S. faecalis and P. cerevisiae, most likely due to a small amount of contamination by peaks VIII and X. Peak X was identified as H_4PteGlu because of its lability to heat and light, and its elution order on the DEAE-cellulose column (based on the elution order of reduced monoglutamates). For a more positive confirmation of these identifications, co-chromatography with synthetic standards for 5-CHO-,

Table 3. Microbiological Assay of Individual Folate Peaks Obtained from DEAE-Cellulose Column Chromatography of Rat Liver Extracts

	L. casei			S. faecalis			P. cerevisiae	
DEAE peak	No. of columns assayed	B.C.[1]	A.C.[2]	No. of columns assayed	B.C.	A.C.	No. of columns assayed	A.C.
Total I-X	5	6.13	14.60					
VII[3]	7	0.17	0.33	6	0.014	0.20	2	0.14
VIII	7	0.83	1.88	6	0.011	1.59	2	2.35
IX	7	2.70	6.45	5	0.010	1.18	2	1.48
X	7	1.30	4.69	6	0.008	1.56	1	2.10
Total VIII-X	7	4.90	13.35					

[1]Microbiological assay values (μg/g liver) before conjugase treatment(B.C.)
[2]Microbiological assay values (μg/g liver) after conjugase treatment(A.C.)
[3]These numbers refer to combined fractions of peaks shown in Fig. 1.

5-CH_3-H_4PteGlu$_5$ and H_4PteGlu$_5$, plus extensive microbiological assays of all column fractions, are required.

In summary, the data in Figs. 1 and 2 and Table 3 show that 85-90% of liver folates in rats are pentaglutamate forms of folic acid. These results clearly establish the importance of polyglutamates as major forms of liver folates.

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