Distribution of Pteroylglutamates in Rat Liver During Regeneration after Partial Hepatectomy

Mario MARCHETTI, Brunella TOLOMELLI, Gabriella FORMIGGINI and Carla BOVINA
Istituto di Chimica Biologica dell'Università di Bologna, Via Irnerio 48, 40126 Bologna, Italy

and Bruno BARBIROLI
*Istituto di Chimica Biologica dell'Università di Modena, Via Campi 278,
41100 Modena, Italy*

(Received 31 January 1980)

1. Liver pteroylpolyglutamate distribution was studied during regeneration after partial hepatectomy in rats maintained under controlled feeding conditions. 2. Pteroylhexaglutamate, pteroylpentaglutamate and pteroyltetraglutamate concentrations decrease from 12 to 72 h after operation, then increase and reach normal values at 180 h. Pteroyltriglutamate concentration, already high at 12 h, remains so in the subsequent periods. Pteroyldiglutamate concentration was unchanged. Monoglutamate concentrations at first decrease, and at 180 h exceed normal values. 3. The decrease in polyglutamate derivatives with a high number of glutamate residues, at present considered to be the coenzyme forms of folate, could be related not to a decreased synthesis, but to a greater requirement for these compounds during the early periods of regeneration, when biosynthetic processes are markedly increased. It is indeed probable that the increased availability of the preferred substrate of pteroylpolyglutamate synthetase, i.e. tetrahydrofolate, enhances conversion of folate into coenzyme forms.

In the course of our studies on the regulatory mechanism of biosynthesis and utilization of folate coenzymes, we have investigated folate metabolism in rapidly growing tissues, as these tissues require a larger availability of folate coenzymes, essential as C₁ donors in the synthesis of purine and pyrimidine nucleotides and in the metabolism of some amino acids (Blakley, 1969; Marchetti, 1971).

The developing chick embryo showed a constant increase in these coenzymes during growth, particularly on day 12, when biosynthetic processes are more active. The contemporaneous increase in enzymic activities involved in their biosynthesis led us to assume that the larger demand could induce these enzymes to synthesize the suitable coenzymic forms (Landi *et al.*, 1972).

In the regenerating rat liver after partial hepatectomy, significant variations in folate derivatives were found, especially in the concentration of the reduced forms. In this experimental condition the enzyme activities involved in folate synthesis were also found to be significantly increased (Barbiroli *et al.*, 1975).

Abbreviations used: pteroylglutamates are indicated by the abbreviations PteGlu, PteGlu_n, PteGlu_n, etc., the subscript numerals indicating the number of glutamate residues present.

Monoglutamate derivatives have previously been regarded as the more active forms of folate, whereas polyglutamates, the major part of the total pool of folates in animal tissues (Bird *et al.*, 1965; Shin *et al.*, 1972; Houlihan & Scott, 1972; Osborne-White & Smith, 1973), have been regarded as the storage forms of the vitamin.

There is now considerable evidence that the coenzyme form of folate in mammalian cells is the polyglutamate derivative. In fact studies performed *in vitro* by testing some polyglutamates and monoglutamates in folate-dependent reactions have shown that each polyglutamate derivative is more active than the corresponding monoglutamate (Coward *et al.*, 1975; Cheng *et al.*, 1975). This has been shown also in experiments *in vivo* (Lavoie *et al.*, 1975).

Therefore pteroylpolyglutamates must now be regarded as the functional coenzymic form of folate. On the other hand, monoglutamate derivatives must be regarded as transport forms of the vitamin (Hoffbrand *et al.*, 1973; Shane & Stokstad, 1976), whereas di- and tri-glutamate derivatives probably occur only as intermediates in the process of adding glutamate residues to monoglutamates (Leslie & Baugh, 1974). In view of all these observations the synthesis of polyglutamate derivatives may rep-

resent another important regulatory step in the folate-dependent processes.

In the present paper we report the results of experiments on folate polyglutamate distribution in the rat liver during regeneration after partial hepatectomy. These experiments have been performed on rats maintained under controlled feeding conditions, to evaluate more precisely the quantitative phenomena caused by the regeneration. In fact, it has been shown that rats exhibit daily rhythms of the enzyme activities involved in folate metabolism (Barbiroli *et al.*, 1974).

Experimental

Animals

Male albino rats of Wistar strain (30 days old) weighing 80–90 g were housed, usually in pairs, in an air-conditioned windowless room lit from 21:00 to 09:00 h. Food, a laboratory diet containing folate (10 mg/kg of diet), was supplied just before the lights were switched off and was removed 8 h later, according to the '8 + 16' feeding schedule developed by Potter (1970). Water was supplied *ad libitum*.

The animals were maintained under these controlled conditions for 4 weeks, by which time partial hepatectomy was performed at 21:00 h $\pm$ 30 min under diethyl ether anaesthesia, with removal of the main lobes (68–70% of liver) as described by Higgins & Anderson (1931).

The portions of liver excised were rinsed in ice-cold 0.9% NaCl, blotted on filter paper, promptly frozen and stored at -80°C . These pieces were used to evaluate folate content in the livers at zero time.

At intervals of 12, 24, 36, 72, 120 and 180 h after operation, the rats were killed by decapitation and the livers were quickly removed and subjected to the same treatment.

Separation and determination of folates

The livers were minced and immediately dropped into 4 vol. of hot (95°C) 1.1% sodium ascorbate,

pH 6, and boiled for 5 min in a water bath. After cooling, the whole mixture was homogenized for 60 s and then centrifuged at 12000 g for 10 min at 4°C .

A 2 ml portion of the supernatant, corresponding to 0.4 g of liver, was applied to a Sephadex G-15 column (1.5 cm $\times$ 90 cm) prepared by the method of Shin *et al.* (1975). Elution of poly- and monoglutamates was carried out with 0.025 M-potassium phosphate buffer, pH 7, containing 0.2 M-2-mercaptoethanol; 120 fractions (1.7 ml) were collected at a flow rate of 7 ml/h.

The folate activity in the single fractions was determined by microbiological assay before and after conjugase treatment (Bird *et al.*, 1965). Conjugase was prepared from hog kidney as described by Eignen & Shockman (1963).

Microbiological assay was carried out with *Lactobacillus casei* (A.T.C.C. 7469) by the procedure of Waters & Mollin (1961) with some modification (Bovina *et al.*, 1977; Tolomelli *et al.*, 1977). The identity of each folate compound was determined by its absorption spectrum and by k_{av} values of standard polyglutamates.

Results and Discussion

Fig. 1 shows the elution profiles of pteroylglutamates in rat liver during 7 days of regeneration after partial hepatectomy, and results in Table 1 show the liver contents of the single forms of polyglutamates in the same experimental conditions. PteGlu₅ and PteGlu₆ concentrations showed an almost constant decrease between 12 and 72 h after partial hepatectomy, whereas from 72 to 180 h they reached normal values again. Also PteGlu₄ concentrations show a very similar behaviour. PteGlu₃ concentration was found to increase after 12 h, and remained high in the subsequent times studied. PteGlu₂ concentration did not show a significant variation. PteGlu concentration decreased during the early times of regeneration, whereas afterwards it reached the zero-time values and even exceeded them.

Table 1. Polyglutamate forms of folate in rat liver during regeneration after partial hepatectomy
Separation and determination methods for single folate forms are described in the Experimental section. Results represent the means for four determinations performed on pooled livers (four to six livers per pool) and are expressed as ng/g wet wt. $\pm$ S.E.M.

Time after operation (h)	Folate forms (ng/g wet wt.)					Polyglutamate/ monoglutamate ratio
	PteGlu ₅₊₆	PteGlu ₄	PteGlu ₃	PteGlu ₂	PteGlu	
0	9830 $\pm$ 875	515 $\pm$ 106	1995 $\pm$ 138	220 $\pm$ 29	1450 $\pm$ 107	6.78
12	9019 $\pm$ 714	407 $\pm$ 78	2225 $\pm$ 196	190 $\pm$ 31	1127 $\pm$ 122	7.76
24	6980 $\pm$ 625	287 $\pm$ 74	3227 $\pm$ 278	290 $\pm$ 17	780 $\pm$ 95	8.94
36	6230 $\pm$ 640	314 $\pm$ 95	2473 $\pm$ 223	174 $\pm$ 27	711 $\pm$ 63	8.76
72	5250 $\pm$ 427	307 $\pm$ 83	2550 $\pm$ 236	220 $\pm$ 33	950 $\pm$ 118	5.53
120	7932 $\pm$ 680	428 $\pm$ 125	2735 $\pm$ 187	253 $\pm$ 21	1319 $\pm$ 235	5.22
180	8980 $\pm$ 775	467 $\pm$ 117	2580 $\pm$ 275	314 $\pm$ 43	1865 $\pm$ 212	4.80

Fig. 1. Elution profiles of pteroylglutamates in rat liver during regeneration after partial hepatectomy

Folate derivatives were extracted from the liver at various time intervals after the operation, separated on a Sephadex G-15 column by the method of Shin *et al.* (1975) and quantitatively evaluated as detailed in the Experimental section. Numbers by the arrows indicate the numbers of glutamate residues in the eluted peak. Numbers on the right-hand side of each elution profile indicate the time interval (hours) after the operation.

The first noteworthy result of this research is the considerable decrease in liver higher polyglutamate forms of folate during the early phases of regeneration. Such a decrease can be related not to a lower synthesis, but to a greater utilization of these compounds when biosynthetic processes are largely increased, as is the case during the early periods of regeneration.

It is known that liver DNA synthesis is at a maximum at 21h after partial hepatectomy in rats maintained under our experimental conditions (Barbiroli & Potter, 1971), and it is well known that this synthesis, as well as RNA and protein synthesis, are folate-dependent processes.

The great demand of these coenzymes can increase the conversion of mono- into poly-glutamates by stimulating pteroylpolyglutamate synthetase activity. This is suggested not only by the increase in PteGlu₃ concentration, an intermediate in the polyglutamate synthetic pathway (Leslie & Baugh, 1974), but also by the decrease in monoglutamates. Consequently, the polyglutamate/monoglutamate ratio is very high at 24h, higher than that observed at zero time (see Table 1).

Pteroylpolyglutamate synthesis in regenerating liver is favoured by a larger availability of particular monoglutamates, which are direct precursors in this synthesis. In fact it was demonstrated by experiments *in vitro* that pteroylpolyglutamate synthetase utilizes reduced rather than oxidized forms as preferred substrates. Among the reduced forms, tetrahydrofolate seems to be the preferred one, whereas 5-methyltetrahydrofolate is utilized only in very small amounts (Masurekar & Brown, 1975; Ritari *et al.*, 1975; Hoffbrand *et al.*, 1976).

This is also supported by experiments performed *in vivo* in different experimental conditions, i.e. (a) in vitamin B₁₂-deficiency a decrease in liver pteroylpolyglutamate synthesis is attributed to an increase in 5-methyltetrahydrofolate at the expense of other reduced forms (Shin *et al.*, 1975); (b) in methotrexate-treated rats the same result is obtained by inhibiting the synthesis of tetrahydrofolates (Brown *et al.*, 1974).

In regenerating liver, the biosynthetic process can be supported by the increase in tetrahydrofolate synthesis and by the contemporaneous decrease in methyltetrahydrofolate content (Barbiroli *et al.*, 1975).

Our present findings suggest the presence of a regulatory mechanism of folate coenzyme availability depending on cellular requirements. Such a mechanism would be based on the regulation of enzyme activities involved in: (1) the reduction of folate to tetrahydrofolate; (2) the attachment of various C₁ units; (3) the addition of γ -glutamate residues.

References

- Barbiroli, B. & Potter, V. R. (1971) *Science* **172**, 738–741
- Barbiroli, B., Bovina, C., Tolomelli, B. & Marchetti, M. (1974) *Proc. Soc. Exp. Biol. Med.* **145**, 645–647
- Barbiroli, B., Bovina, C., Tolomelli, B. & Marchetti, M. (1975) *Biochem. J.* **152**, 229–232

- Bird, O. D., McGlohon, V. M. & Vaitkus, J. W. (1965) *Anal. Biochem.* **12**, 18–35
- Blakley, R. L. (1969) in *The Biochemistry of Folic Acid and Related Pteridines* (Neuberger, A. & Tatum, E. L., eds.), North-Holland, Amsterdam
- Bovina, C., Tolomelli, B. & Formiggini, G. (1977) *Boll. Soc. Ital. Biol. Sper.* **53**, 2099–2103
- Brown, J. P., Davidson, G. E., Weir, D. G. & Scott, J. M. (1974) *Int. J. Biochem.* **5**, 727–733
- Cheng, F. W., Shane, B. & Stokstad, E. L. R. (1975) *Can. J. Biochem.* **153**, 1020–1027
- Coward, J. K., Chello, P. L., Cashmore, A. R., Parameswaran, K. N., De Angelis, L. M. & Bertino, J. R. (1975) *Biochemistry* **14**, 1548–1552
- Eignen, E. & Shockman, G. D. (1963) in *Analytical Microbiology* (Kavanagh, F., ed.), pp. 431–488, Academic Press, New York
- Higgins, G. M. & Anderson, R. M. (1931) *Arch. Pathol.* **12**, 186–202
- Hoffbrand, A. V., Tripp, E., Houlihan, C. M. & Scott, J. M. (1973) *Blood* **42**, 141–146
- Hoffbrand, A. V., Tripp, E. & Lavoie, A. (1976) *Clin. Sci. Mol. Med.* **50**, 61–68
- Houlihan, C. M. & Scott, J. M. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1675–1681
- Landi, L., Pasquali, P. & Marchetti, M. (1972) *Proc. Soc. Exp. Biol. Med.* **141**, 173–175
- Lavoie, A., Tripp, E., Parsa, K. & Hoffbrand, A. V. (1975) *Clin. Sci. Mol. Med.* **48**, 67–73
- Leslie, G. I. & Baugh, C. M. (1974) *Biochemistry* **13**, 4957–4961
- Marchetti, M. (1971) *Acta Vitaminol. Enzymol.* **25**, 41–64
- Masurekar, M. & Brown, G. M. (1975) *Biochemistry* **14**, 2424–2430
- Osborne-White, W. S. & Smith, R. M. (1973) *Biochem. J.* **136**, 265–278
- Potter, V. E. (1970) *Miami Winter Symp.* **2**, 241–313
- Ritari, S. J., Sakami, W., Black, C. W. & Rzepka, J. (1975) *Anal. Biochem.* **63**, 118–129
- Shane, B. & Stokstad, E. L. R. (1976) *J. Biol. Chem.* **251**, 3405–3410
- Shin, Y. S., Williams, M. A. & Stokstad, E. L. R. (1972) *Biochem. Biophys. Res. Commun.* **47**, 35–43
- Shin, Y. S., Buehring, K. U. & Stokstad, E. L. R. (1975) *Mol. Cell. Biochem.* **9**, 97–108
- Tolomelli, B., Bovina, C. & Formiggini, G. (1977) *Boll. Soc. Ital. Biol. Sper.* **53**, 2104–2109
- Waters, A. H. & Mollin, D. L. (1961) *J. Clin. Pathol.* **14**, 335–344