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Distribution of Pteroylglutamates during Liver Regeneration after Partial Hepatectomy in Folic Acid-Deficient Rats (40934)

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Abstract. Normal and folate-deficient rats were partially hepatectomized and sacrificed at various times after the operation. The distribution of pteroylmono- and polyglutamates was evaluated by Sephadex G-15 chromatography and microbiological assay. The incorporation of thymidine into DNA was also studied. Deficient rats show a fall in penta- and hexaglutamates and intermediate forms beginning 12 hr after the operation. Monoglutamates diminished but less than the other compounds. In control rats the higher polyglutamates after an initial decrease rise to normal values at 180 hr; the intermediate forms had already increased at 12 hr and remained high at the following times and monoglutamates at first decreased but at 180 hr exceeded normal values. The rate of DNA synthesis is not only decreased, but also delayed in regenerating liver of deficient rats as compared with controls. The livers of deficient rats appear unable to synthesize adequate quantities of polyglutamates. This fact may explain the slowing down of the regenerative process in folic acid-deficient rats, demonstrated by lower and delayed thymidine incorporation into DNA.

A great number of metabolic processes in which C_1 units are involved, i.e., purine and pyrimidine nucleotide synthesis and amino acid interconversion, are dependent on folate coenzymes (1, 2). Therefore, it seems that their availability should be regulated in the cell according to the rate of nucleic acid and protein synthesis.

Our interest has been directed toward the study of these regulatory mechanisms under experimental conditions in which these biosynthetic processes reach high rates. In this regard it is interesting to report that the administration of testosterone or 17β-estradiol to castrated male or female rats, respectively, normalized the enzymic activities involved in the conversion of folates into their coenzymic forms, which dropped after castration (3–9). Similarly, in developing chick embryo (10) and in regenerating rat liver following partial hepatectomy (11), we have found a close relationship between the increased biosynthetic processes and folate coenzyme synthesis.

These results suggest that the synthesis of folate coenzymes, and consequently their availability, are regulated by the actual requirement for biosynthesis of DNA, RNA, and proteins.

It is now well known that the conversion of folic acid in active coenzymic forms consists not only in the reduction of the vitamin and in the introduction of C_1 units at different oxidation levels, but also in the addition of conjugated γ-glutamyl residues. In fact, research performed *in vitro* (12, 13) and *in vivo* (14) has shown that the active coenzymic folate forms are the polyglutamate ones, especially with a high number of glutamyl residues. On the other hand, these compounds constitute the major part of the folate pools in animal tissues (15–17). Moreover, they are more easily retained by tissues as they do not readily cross cell membranes (18, 19).

Therefore, it is quite clear that the process of conversion of monoglutamate forms into the polyglutamate ones is now considered very important in terms of regulation of folate-dependent processes.

In the present paper we report experiments undertaken to investigate the influence of folic acid on the process of liver

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regeneration following partial hepatectomy by studying the distribution of polyglutamates and the rate of DNA synthesis in folic acid-deficient rats. The animals were maintained under controlled feeding conditions to evaluate more precisely the quantitative phenomena caused by regeneration (20).

Materials and methods. Male Wistar albino rats, 30 days old, weighing 70 to 80 g, were employed in this experiment.

The animals were divided into two groups, according to diet. Group 1 was fed on a folic acid-free diet consisting of (g/kg) corn flour, 790; vitamin-free casein, 120; vegetable oil, 40; salt mixture, 40;[2] succinylsulfathiazole, 10; and the following vitamins (mg/kg): thiamine, 4; riboflavin, 3; pyridoxine, 1; niacin, 50; calcium pantothenate, 16; choline chloride, 1000. Vitamin A (2500 units) and vitamin D (500 units) were dissolved in the vegetable oil.

Group 2 was fed the same diet supplemented with folic acid (10 mg/kg).

The animals were housed in pairs in wire-floored cages to prevent coprophagy, in a constant temperature, windowless room illuminated from 21:00 to 09:00 hr. Food was supplied just before the lights were switched off and was removed 8 hr later according to the feeding schedule developed by Potter (21). 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 ± 30 min under ether anesthesia, with removal of the main lobes (68–70% of the liver) as described by Higgins and Anderson (22).

The portions of liver excised were rinsed in ice-cold 0.9% saline, plotted on filter paper, promptly frozen, and stored at −80°. These pieces were employed to evaluate folates present in the livers at zero time.

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

Separation and estimation of folates. The livers, finely divided into small pieces, were immediately dropped into 4 vol of hot (95°) 1.1% Na-ascorbate, pH 6, and boiled for 5 min in a water bath. After cooling, the whole mixture was homogenized for 60 sec and then centrifuged at 12,000*g* for 10 min at 4°.

An aliquot of 2 ml of supernatant, corresponding to 0.4 g of liver, was applied to a 1.5 × 90-cm Sephadex G-15 column prepared according to Shin *et al.* (23). Elution of the derivatives, poly- and monoglutamate folates, was carried out with 0.025 *M* K-phosphate buffer, pH 7, containing 0.2 *M* mercaptoethanol; 120 fractions of 1.7 ml were collected at a flow rate of 7 ml/hr.

The folic acid activity in the single fractions, as well as in the unchromatographated liver extract, was determined by microbiological assay before and after being treated with conjugase. The conjugase preparation was made from hog kidney as described by Eignen and Shockman (24), and enzymic treatment of the samples was done according to the method of Bird *et al.* (15).

The microbiological assay was carried out with *Lactobacillus casei* (ATCC 7469) according to the procedure of Waters and Mollin (25) with some modifications (26, 27). The identity of each folate compound separated by chromatography and microbiologically valued was carried out by determination of its absorption spectrum and by K_{av} values of standard polyglutamates.

Incorporation of thymidine into DNA. To evaluate DNA synthesis, eight rats of each group were injected intraperitoneally with 20 μCi/100 g body wt of [*methyl*-^{3}H]-thymidine (Radiochemical Centre Amersham, Buchs, England; sp act 20 Ci/mmole) in 0.4 ml of saline at intervals ranging between 15 and 37 hr after partial hepatectomy and killed by decapitation exactly 1 hr after the injection. The livers, quickly removed, dropped into cold saline, and blotted with filter paper, were homogenized in 9 vol of distilled water. Perchloric acid was added to an aliquot of the homogenate to a final molarity of 0.5.

[2] Ca-lactate, 35.15; $Ca(H_2PO_4)_2 \cdot H_2O$, 14.60; K_2HPO_4, 25.78; $NaH_2PO \cdot H_2O$, 9.38; NaCl, 4.67; $MgSO_4$, 7.19; Fe citrate, 3.19.

After centrifugation the supernatant was saved and the residue was washed once with cold 0.5 *M* perchloric acid.

The supernatants were combined and designated the "acid-soluble fractions." The DNA in the pellet was separated from the RNA (28) and was assayed by the Ceriotti (29) procedure slightly modified (30). Radioactivity was measured on a Packard Tri-Carb liquid scintillation spectrometer.

Results. The data reported in Table I show that total folate activity (*L. casei*) in deficient rat liver, which as expected was very low at zero time as compared with the control rats, considerably decreased during the first 12 hr of regeneration. These low values have also been observed at all the following times studied. Control rats also showed a considerable decrease of liver total folate activity during the early phases of regeneration but, unlike deficient animals, this activity tended to equal the zero time values 120 hr after the operation.

Figures 1 and 2 show the elution patterns of the folate derivatives, and Table II gives the amounts of these compounds expressed as nanograms per gram of wet tissue.

In folic acid-deficient rats hexa- ($PteGlu_6$) and pentaglutamates ($PteGlu_5$) show a remarkable fall by 12 hr after hepatectomy and they are also present in very low quantities at 24, 36, and 72 hr. After this time there is a slight increase, but the values are clearly lower than those found at zero time. As far as the intermediate forms are concerned: tetraglutamates ($PteGlu_4$) and diglutamates ($PteGlu_2$) diminish after the operation and they do not increase at the following times studied; triglutamates ($PteGlu_3$), after an initial fall, slightly increase in the subsequent periods. Also, monoglutamate forms appear to drop in the early phases of regeneration and subsequently show an increase.

In the control animals the higher polyglutamates ($PteGlu_6$, $PteGlu_5$) show a constant decrease from 12 to 72 hr after hepatectomy and a rise up to normal values at 180 hr; tetraglutamates ($PteGlu_4$) exhibit similar behavior. The triglutamates, already increased at 12 hr, also remain high at the following times, while diglutamates do not show any significant variation. Finally,

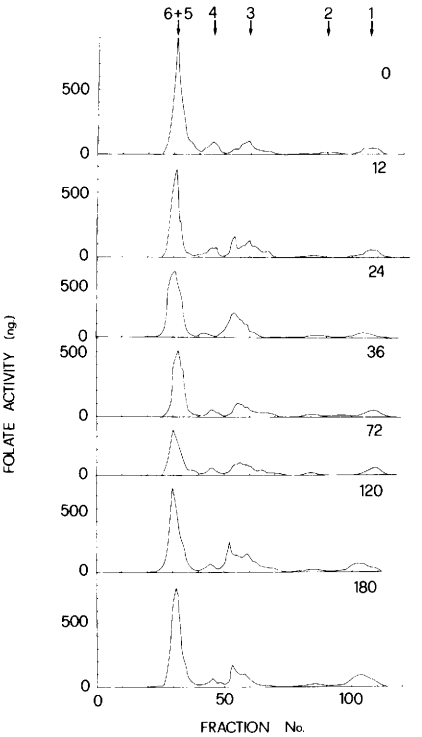

FIG. 1. Pattern of liver folate derivatives in control rats during regeneration following partial hepatectomy. Single compounds were separated on a Sephadex G-15 column and quantitatively evaluated by microbiological assays performed on the single fractions as specified under Material and Methods. The numbers over the arrows show the number of glutamate moieties. The number on the right-hand side of each graph indicates the hours after partial hepatectomy.

TABLE I. TOTAL FOLATE ACTIVITY OF RAT LIVER DURING REGENERATION AFTER PARTIAL HEPATECTOMY

Time after operation (hr)	Experimental animals	
	Control	Deficient
0	14,532 ± 715	1,680 ± 183
12	13,350 ± 613	1,080 ± 92
24	11,490 ± 607	960 ± 103
36	10,351 ± 585	850 ± 84
72	9,174 ± 562	890 ± 97
120	13,200 ± 710	960 ± 105
180	15,105 ± 822	1,090 ± 118

Note. The data represent the means ± SEM of eight determinations and are expressed as ng/g wet wt.

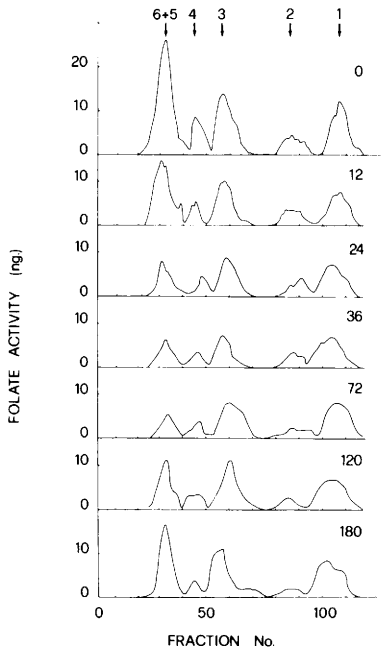

FIG. 2. Pattern of liver folate derivatives in folic acid-deficient rats during regeneration following partial hepatectomy. Single compounds were separated on a Sephadex G-15 column and quantitatively evaluated by microbiological assays performed on the single fractions as specified under Material and Methods. The numbers over the arrows show the number of glutamate moieties. The number on the right-hand side of each graph indicates the hours after partial hepatectomy.

monoglutamate forms show a fall in the early phases of regeneration and then they increase and at 180 hr they reach values higher than those at zero time.

Figure 3 shows the incorporation of labeled thymidine into DNA at different times after the partial hepatectomy. The data are reported as percentage of total uptake, defined as the sum of the radioactivities in DNA and in the acid-soluble fraction at 1 hr after injection. This way of expressing the data has been chosen to minimize the possible variation due to the transport of the labeled precursor into the liver cell.

Deficient rats show that 21 hr after partial hepatectomy the incorporation rate of thymidine into DNA is very low, while in the control rats its level has already reached a maximum. In deficient rats the first peak of DNA synthesis is reached with a 4-hr delay and it is considerably lower than that of control rats.

Discussion. In order to verify the regeneration process in folic acid deficiency we have studied the rate of DNA synthesis, during the early phases, by measuring the incorporation of its labeled precursor thymidine. During the times studied it appears that the rate of DNA synthesis is not only decreased but also delayed. In fact, 21 hr after the operation thymidine incorpora-

TABLE II. POLYGLUTAMATE FORMS OF FOLATE OF LIVER DURING REGENERATION AFTER PARTIAL HEPATECTOMY

Experimental animals	Time after operation (hr)	Folate compounds (ng/g wet wt)				
		$PteGlu_{6+5}$	$PteGlu_4$	$PteGlu_3$	$PteGlu_2$	$PteGlu_1$
Control	0	9830 ± 875	515 ± 106	1995 ± 138	220 ± 29	1350 ± 107
	12	9420 ± 714	407 ± 78	2225 ± 196	190 ± 31	1227 ± 122
	24	7980 ± 625	287 ± 74	3227 ± 278	290 ± 17	780 ± 95
	36	6530 ± 640	314 ± 95	2973 ± 223	174 ± 27	611 ± 63
	72	5250 ± 427	267 ± 83	2550 ± 236	220 ± 33	850 ± 118
	120	7930 ± 680	528 ± 125	2735 ± 187	253 ± 21	1519 ± 235
	180	8980 ± 775	667 ± 117	2580 ± 275	314 ± 43	1865 ± 212
Deficient	0	543 ± 92	121 ± 15	318 ± 52	138 ± 15	268 ± 35
	12	281 ± 34	61 ± 8.1	205 ± 34	92 ± 11	165 ± 22
	24	108 ± 12	65 ± 6.1	188 ± 25	74 ± 8.5	189 ± 23
	36	92 ± 9.2	49 ± 6.7	167 ± 14	71 ± 7.1	197 ± 21
	72	116 ± 14	52 ± 5.3	208 ± 19	53 ± 6.9	225 ± 23
	120	171 ± 25	63 ± 7.1	227 ± 23	49 ± 5.2	221 ± 32
	180	265 ± 27	52 ± 7.9	258 ± 25	32 ± 4.3	245 ± 54

Note. The values represent the means ± SEM of four determinations of pools of two livers.

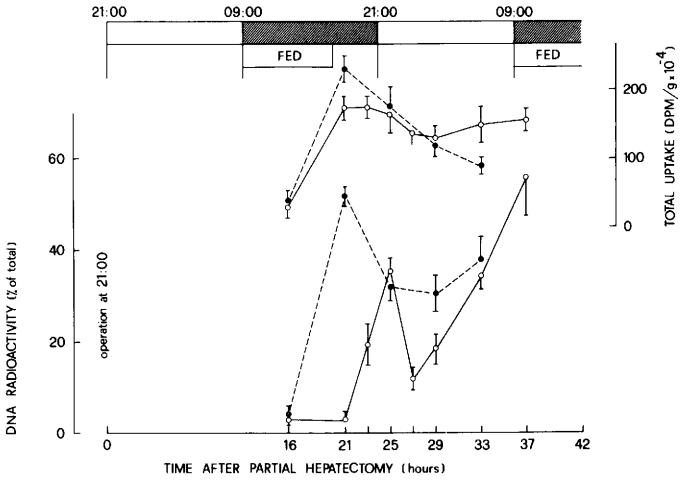

FIG. 3. Incorporation of labeled thymidine into liver DNA at different times after partial hepatectomy. The cross-hatched sections represent the dark period in the 24-hr cycles and the open sections below them represent the periods when food was available. The data are reported as a percentage of total uptake, defined as the sum of the radioactivities in the DNA and soluble fractions at 1 hr after the injection of the label. Upper curves, total uptake; lower curves, percentage in DNA. Broken lines, control rats; solid lines, folic acid-deficient rats. Each point represents the mean of six rats ± SEM indicated by vertical bars.

tion into DNA is still practically undetectable while the control rats show a very high level of incorporation.

Large differences have also been found in the total amount of liver folates and even more in the behavior of the single forms.

As far as folate derivatives as a group are concerned, lower total folate activity was observed beginning at the early phases of regeneration in both groups of animals. However, while in deficient rats the values were also low at the following times studied, in control rats there was a rise to normal values 120 hr after the operation.

TABLE III. RATIO OF HIGHER POLYGLUTAMATE/MONOGLUTAMATE DERIVATIVES IN LIVER DURING REGENERATION AFTER PARTIAL HEPATECTOMY

Time after operation (hr)	Experimental animals	
	Control	Deficient
0	7.28	2.03
12	7.67	1.70
24	10.23	0.57
36	10.68	0.47
72	6.18	0.51
120	5.22	0.77
180	4.81	1.08

Among the single compounds, in the deficient rats we observed a greater fall of higher polyglutamates, as compared with that of mono- and triglutamates. However, in normal animals the higher polyglutamates show a smaller decrease than the monoglutamates. Consequently the poly/monoglutamate ratio fell in deficient rats, while it rose in controls during the initial times of regeneration (Table III).

Based on the reports (12–14) that polyglutamates with a higher number of γ-glutamyl residues are the real coenzymic forms of folic acid, the decrease of these compounds in the regenerating livers of both groups of animals must be ascribed to the higher utilization in those biosynthetic processes which are, in this experimental condition, very exalted. However, while in control animals this higher coenzyme requirement is, almost partially, satisfied by an increase of their biosynthesis, utilizing the available monoglutamates, this does not occur in deficient rats. In fact, in these animals there is an almost complete disappearance of higher polyglutamate forms in spite of the presence of monoglutamates. It is possible that in deficient rat liver there is a

defective conversion from monoglutamate forms. This, in turn, may be ascribable to the lower activity of pteroylpolyglutamate synthetase and/or to the lower availability of the reduced forms of folic acid, which are the preferred substrates for this enzyme (31–33). In fact, in the livers of folic acid-deficient rats a marked decrease in these reduced forms, chiefly tetrahydrofolate, imputable to a modification of enzymic activities involved in their synthesis, has been observed (11).

The lower availability of pteroylpolyglutamates may explain why the only folic acid-dependent process we have studied, i.e., DNA synthesis, is slowed down. However, it must be pointed out that DNA synthesis is preceded by specific and abundant RNA and protein biosynthesis in the G 1 period of the cell replication cycle, both processes being dependent on the availability of active folates.

1. Blakley, R. L., *in* "The Biochemistry of Folic Acid and Related Pteridines" (A. Neuberger and E. L. Tatum, eds.), p. 219. North-Holland, Amsterdam (1969).
2. Marchetti, M., Acta Vitaminol. Enzymol. **25**, 41 (1971).
3. Rovinetti, C., Bovina, C., Tolomelli, B., and Marchetti, M., Biochem. J. **126**, 291 (1972).
4. Bovina, C., Tolomelli, B., Rovinetti, C., and Marchetti, M., Proc. Soc. Exp. Biol. Med. **140**, 176 (1972).
5. Tolomelli, B., Rovinetti, C., Bovina, C., and Marchetti, M., Experientia **28**, 197 (1972).
6. Bovina, C., Tolomelli, B., Rovinetti, C., and Marchetti, M., J. Endocrinol. **54**, 457 (1972).
7. Bovina, C., Tolomelli, B., Rovinetti, C., and Marchetti, M., Intern. J. Vit. Nutr. Res. **41**, 453 (1971).
8. Laffi, R., Tolomelli, B., Bovina, C., and Marchetti, M., Intern. J. Vit. Nutr. Res. **42**, 196 (1972).
9. Tolomelli, B., Bovina, C., Rovinetti, C., and Marchetti, M., Proc. Soc. Exp. Biol. Med. **141**, 436 (1972).
10. Landi, L., Pasquali, P., and Marchetti, M., Proc. Soc. Exp. Biol. Med. **141**, 173 (1972).
11. Barbiroli, B., Bovina, C., Tolomelli, B., and Marchetti, M., Biochem. J. **152**, 229 (1975).
12. Cheng, F. W., Shane, B., and Stokstad, E. L. R., Canad. J. Biochem. **153**, 1020 (1975).
13. Coward, J. K., Chello, P. L., Cashmore, A. R., Parameswaran, K. N., De Angelis, L. M., and Bertino, J. R., Biochemistry **14**, 1548 (1975).
14. Lavoie, A., Tripp, E., Parsa, K., and Hoffbrand, A. V., Clin. Sci. Mol. Med. **48**, 67 (1975).
15. Bird, O. D., McGlohon, V. M., and Vaitkus, J. W., Anal. Biochem. **12**, 18 (1965).
16. Houlihan, C. M., and Scott, J. M., Biochem. Biophys. Res. Commun. **48**, 1675 (1972).
17. Osborne-White, W. S., and Smith, R. M., Biochem. J. **136**, 265 (1973).
18. McBurney, M. W., Whitmore, G. F., Cell **2**, 173 (1974).
19. Shane, B., and Stokstad, E. L. R., J. Biol. Chem. **250**, 2243 (1975).
20. Barbiroli, B., Bovina, C., Tolomelli, B., and Marchetti, M., Proc. Soc. Exp. Biol. Med. **145**, 645 (1974).
21. Potter, V. R., Miami Winter Symp. **2**, 241 (1970).
22. Higgins, G. M., and Anderson, R. M., Arch. Pathol. **12**, 186 (1931).
23. Shin, Y. S., Buehring, K. U., and Stokstad, E. L. R., J. Biol. Chem. **247**, 7266 (1972).
24. Eignen, E., and Shockman, G. D., *in* "Analytical Microbiology" (F. Kavanagh, ed.), p. 448. Academic Press, New York (1963).
25. Waters, A. H., and Mollin, D. L., J. Clin. Pathol. **14**, 335 (1961).
26. Bovina, C., Tolomelli, B., and Formiggini, G., Boll. Soc. It. Biol. Sper. **53**, 2099 (1977).
27. Tolomelli, B., Bovina, C., and Formiggini, G., Boll. Soc. It. Biol. Sper. **53**, 2104 (1977).
28. Munro, H. N., and Fleck, A., Methods Biochem. Anal. **14**, 113 (1966).
29. Ceriotti, G., J. Biol. Chem. **198**, 297 (1952).
30. Blobel, G., and Potter, V. R., **154**, 1662 (1966).
31. Masurekar, M., and Brown, G. M., Biochemistry **14**, 2424 (1975).
32. Ritari, S. J., Sakami, W., Black, C. W., and Rzepka, J., Anal. Biochem. **63**, 118 (1975).
33. Hoffbrand, A. V., Tripp, E., and Lavoie, A., Clin. Sci. Mol. Med. **50**, 61 (1976).

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