The Turnover of Rat-Liver Folate Pools[1] (37084)

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The folate forms present in tissue have been difficult to investigate for several reasons. Tissue folates cannot be isolated in their natural state unless the highly active γ-glutamyl carboxypeptidase (conjugase) is inactivated immediately after disruption of the tissue and unless oxidation of the highly labile folates is prevented by the use of reducing compounds. The analysis procedure of microbiological assay used along with conjugase treatment provides little information concerning the number of glutamate residues in folate polyglutamates. Also, the free folates in liver cannot be assayed accurately with *Lactobacillus casei* in the presence of the polyglutamate derivatives (1, 2).

We have overcome some of these difficulties by developing a method using column chromatography following injection of tritiated folic acid (3). In addition, rat liver folates have been more specifically identified by gel filtration as 85–90% reduced folate pentaglutamates, 5% monoglutamates and the remainder as di- and triglutamates (4).

Since the function of the pentaglutamate conjugates comprising 85–90% of the total folate in liver is unknown, we have studied these pentaglutamate pools by measuring the uptake of a single intravenous dose of tritiated PteGlu[3] into liver folate pools and its subsequent removal under steady-state conditions in the adult rat. Half-life values were calculated from these data.

Methods and Materials. Eighteen adult male Sprague–Dawley[4] rats weighing 366 ± 9.2 g (standard deviation) were fed a 20% soy protein basal diet (5) plus 0.5% DL-methionine and 100 μg/kg vitamin B_{12}, which provided all the known dietary requirements for the rat. The basal diet had the following composition (g/kg): soy assay protein, 200; glucose monohydrate, 714; corn oil with vitamins A, D, and E, 40; salt mix (6), 35; vitamin premix in glucose monohydrate, 10; choline chloride, 1. The vitamins were supplied in the following amounts (per kg): vitamin A, 15,000 IU; vitamin D (viosterol), 2000 units; α-tocopherol acetate, 50 mg; biotin, 0.2 mg; thiamine HCl, 15 mg; riboflavin, 15 mg; pyridoxine HCl, 15 mg; Ca-pantothenate, 50 mg; niacin HCl, 50 mg; menadione, 10 mg; folic acid, 5 mg. Diet and water were fed *ad libitum* and the animals were housed singly in stainless steel metabolism cages. After the rats were fed this diet for three weeks, each animal was injected with 20 μCi of purified [^{3}H]-PteGlu (40 Ci/mmole)[5] in the femoral vein and returned to the metabolism cages. Urine was collected weekly or at autopsy for determination of radioactivity. Two animals were sacrificed at each of the following time periods after injection: 10, 24, and 48 hr and 4, 7, 14, 21, 28, and 49 days.

At each time interval the rats were killed with chloroform and 6 to 10 ml of blood was withdrawn from the heart with a heparinized syringe for subsequent separation of plasma. The liver was flushed and cooled quickly by perfusing it before removal with 30 ml of 0.9% NaCl at 4°. A 5.0-g portion of the liver was extracted by dropping slices into a tube

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[3] Abbreviations: PteGlu, pteroylglutamate; DE AE-, diethylaminoethyl-; dpm, disintegrations per minute.

[4] Simonson Laboratories, Gilroy, California.

[5] 3′,5′ positions of *p*-aminobenzoate moiety of folic acid labeled with tritium, Amersham/Searle Corporation, Des Plaines, Illinois.

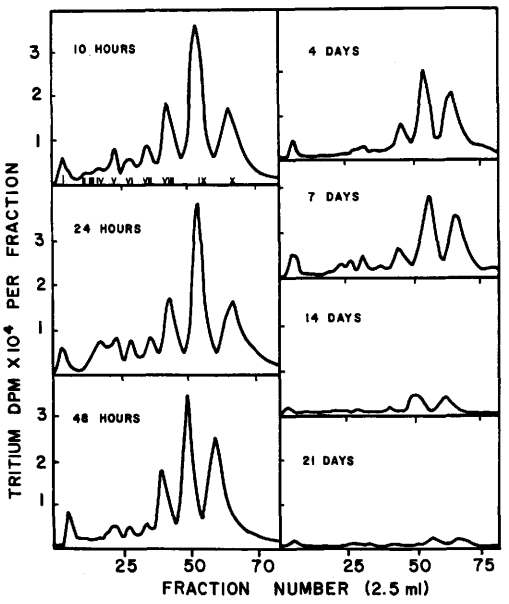

FIG. 1. Elution patterns of tritiated folate pools in rat liver at time intervals to 21 days after iv injection of 20 μCi ^{3}H-PteGlu. Extracts of 1.0 g liver were chromatographed on DEAE-cellulose columns with an exponential phosphate buffer gradient at pH 6.0. Details in text.

containing 20 ml of 1.1% sodium ascorbate solution, pH 6.0 at 95° (7). Heating was continued for 5 min and then the sample was cooled in an ice bath. This extract was homogenized 10 sec with a teflon pestle, glass homogenizer, then centrifuged at 12,000*g* for 10 min at 4°. The supernatant liver extract and the plasma sample were stored frozen at —15° until further assay.

A 5.0-ml portion of a liver extract pool from two animals for each time period was applied to a 0.9 × 20-cm DEAE-cellulose column (3) and chromatographed using an exponential buffer gradient of 50 ml of 0.01 *M* potassium phosphate buffer, pH 6.0 and 250 ml of 0.5 *M* buffer, pH 6.0. All buffers contained 0.2% sodium ascorbate to prevent oxidation of labile folates. One hundred 2.5-ml fractions were collected at a flow rate of 0.67 ml/min. The column and fractions were protected from light.

The elution pattern was determined by counting 0.25 ml of every fraction for radioactivity in 10 ml of scintillation fluid (6 g 2,5-diphenyloxazole, 100 g naphthalene, and 40 g thixotropic gel[6] dissolved in 1 liter 1,4-dioxane). Urine, liver extracts and plasma were counted in a similar manner.

Microbiological activity of liver extracts was measured with *L. casei* after hog kidney conjugase treatment (8).

Results and Discussion. The radioactive elution patterns derived from the DEAE-cellulose column chromatography of extracts of 1.0-g portions of rat liver at various time periods after injection of a single 20 μCi dose of [^{3}H]-PteGlu are shown in Fig. 1. The peaks have been numbered as shown for the 10-hr period and identified according to a previously described procedure (4).

Peaks I and II are breakdown products of folic acid, peaks III to VI contain reduced folate monoglutamates as well as some di- and triglutamates, which are either natural folate forms in rat liver or are formed by cleavage of glutamate residues from polyglutamates during extraction. Peak VII is composed mainly of 10-CHO-$H_4PteGlu_5$ with small amounts of other forms of tri- and diglutamates. Peak VIII contains 10-CHO-$PteGlu_5$, presumably the oxidation product of 10-CHO-$H_4PteGlu_5$, partially mixed with 5-CHO-$H_4PteGlu_5$; peak IX is composed of 5-CH_3-$H_4PteGlu_5$ with some contamination of 5-CHO-$H_4PteGlu_5$ which is probably derived from 10-CHO-$H_4PteGlu_5$ during extraction; peak X contains $H_4PteGlu_5$.

The uptake of tritium-labeled PteGlu was rapid in all fractions and reached a maximum between 24 and 48 hr after injection and then dropped slowly until 21 days, when little measurable radioactivity was found in any fractions. The tracer dose (0.22 μg) was less than 0.5% of the daily dietary intake of folic acid and, therefore, would not affect the amount of folate in liver fractions. It is apparent from the examination of the patterns in Fig. 1 that the folate pools are in a constant state of flux and that the pentaglutamate pools represent the major proportion of the folates present in rat liver. After reaching maximal levels, the radioactivity in

[6] Cab-O-Sil, Packard Instrument Co., Inc., Downers Grove, Illinois.

TABLE I. Radioactivity of Urine, Plasma and Liver at Time Intervals after Intravenous Injection of [^{3}H] PteGlu in the Rat.

Time period after injection	Urinary excretion: No. of rats	Urinary excretion: μCi	Urinary excretion: % dose	No. of rats	Plasma (dpm/ml)	Liver (dpm/ng folate)[a]
10 hr	1	2.7	13	2	22,000	39
24 hr	2	3.4	17	2	13,000	43
48 hr	2	4.8	24	2	10,000	40
1st wk	10	4.8	24	2	15,000	24
2nd wk	8	.67	3.3	2	4,600	5.5
3rd wk	6	.46	2.3	2	4,400	.29
4th wk	4	.26	1.3	2	2,200	—
5th wk	2	.13	.66	—	—	—
6th wk	2	.057	.29	—	—	—
7th wk	2	.037	.19	2	0	—

[a] *L. casei* activity after conjugase treatment.

peaks IX and X is maintained to a greater extent than in other peaks during the first 21 days. This suggests a greater recycling of these folates in the polyglutamate pools rather than introduction of newly synthesized non-labeled pentaglutamates.

In Fig. 2 we have plotted the radioactivity for total folates and the combined fractions of peaks VII, VIII, IX and X as identified in Fig. 1 versus time after injection of [^{3}H]-PteGlu for the extract from 1.0 g of liver. The values obtained at 3-wk periods were omitted because accurate values for each fraction cannot be obtained due to the low radioactivity. Using the least squares method, regression lines for $\log_e$ dpm/g liver were fitted to individual values shown above or below the solid lines. Points above or below the broken lines were not included since radioactivity was still being incorporated into some pools at early time periods. Half-life times were obtained from the slopes. The half-life time for total liver folates was calculated to be 4.0 days; for peak VII, 3.3 days; peak VIII, 3.5 days; peak IX, 4.3 days; and peak X, 4.2 days. The increased half-life of peak IX ($5\text{-}CH_3H_4PteGlu_5$) and peak X ($H_4PteGlu_5$) compared to the total liver folates show that these two fractions are not in rapid equilibrium with other parts of the folate pool and, therefore, are less susceptible to degradation. The total folate in the liver of a 350-g rat is about 200 μg. A half-life of 4.0 days could correspond to a loss of 12.5% per day or 25 μg/day. However, the urinary excretion of folic acid by a rat on a low folate diet is less than 1 μg/day. It is apparent that the folate lost from the liver is degraded eventually either in the liver or in other tissues after redistribution. If this degradation occurs in the liver, then folate in peak IX (mainly $5\text{-}CH_3$-

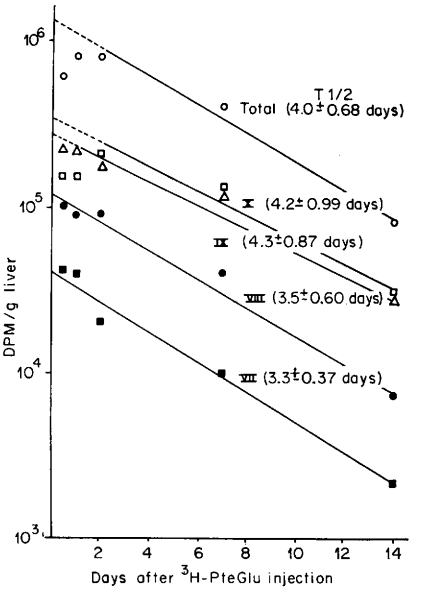

FIG. 2. Turnover of liver folate pools. The half-life time values and the standard errors for total liver folates and for peaks VII to X are given in parentheses. The half-life time for peaks IX and X is significantly different from that for peaks VII and VIII ($p < 0.05$).

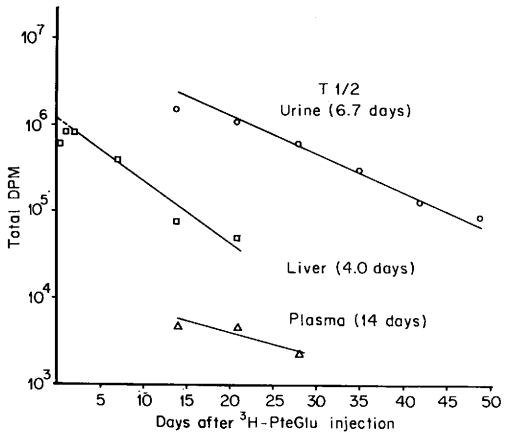

FIG. 3. The disappearance rates of radioactivity in plasma, liver and urine. The half-life time values are given in parentheses and were calculated from the radioactivity in ml samples of plasma and in total liver and urine.

$H_4PteGlu_5$) and X ($H_4PteGlu_5$) must be less susceptible to degradative attack than other folate fractions in liver. A greater half-life for 5-CH_3-$H_4PteGlu_5$ and $H_4PteGlu_5$ compared to that of other peaks (10-CHO-$H_4PteGlu_5$ and others) would require that these two molecular species together with corresponding methylene derivatives be more or less metabolically isolated from the other liver folic acid derivatives (5,10-CH_2-$H_4PteGlu_5$ fraction is included in the $H_4PteGlu_5$ fraction, since 5,10-CH_2-H_4Pte Glu_5 is easily hydrolyzed to $H_4PteGlu_5$ during extraction).

Since the peaks which contain folate monoglutamates were poorly separated from di- and triglutamates and represented only approximately 5% of the total folates, their half-life could not be measured. The disappearance rates of radioactivity in plasma, liver and urine are given in Table I and Fig. 3. The urinary excretion of radioactivity during the 10 hr after injection was 2.7 μCi or 13% of that injected. These values increased to 4.8 μCi or 24% for total excretion for the first week. Its half-life after the first week (6.7 days) was slower than that of liver folates, but faster than that of plasma folates. The plasma radioactivity decreased rapidly until it was equilibrated to a slower rate of decline starting at 2 wk. The half-life time for plasma for the latter time periods was 14 days.

Summary. After a single tracer dose of tritiated pteroylglutamic acid, the uptake, excretion and turnover of radioactivity was measured in the adult rat under steady-state conditions up to 7 weeks after injection. The half-life time of four peaks containing reduced folate pentaglutamates ranged from 3.3 to 4.3 days, indicating a rapid utilization of these liver pools in contrast to the half-life time for plasma radioactivity. The half-life time of the radioactivity excreted in urine is larger than that of liver folates and smaller than that of plasma folates. In view of their rapid turnover and high concentration and variability, it is possible that the pentaglutamate forms in liver serve specific metabolic functions other than simply of a reserve and that folate depletion proceeds at a more rapid rate than previously expected from observations on rates of decrease in plasma folate concentrations.

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