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The Uptake of Pteroyl[^{14}C]glutamic Acid into Rat Liver and Its Incorporation into the Natural Pteroyl Poly-γ-glutamates of that Organ†

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ABSTRACT: The uptake of a dose of pteroyl[U-^{14}C]glutamic acid (Pte-G*) into rat liver and its incorporation into hepatic folates were examined at a number of time intervals from 1 hr to 28 days. The folates were extracted from the liver and then converted quantitatively to the *p*-aminobenzoyl glutamates of corresponding poly-γ-glutamyl chain length. These derivatives were then separated by chromatography on a DEAE-cellulose column using a NaCl gradient in phosphate buffer. Uptake was found to reach a maximum between 6 and 24 hr after intraperitoneal injection of Pte-G*. There was rapid addition of glutamyl residues in the liver so that after 3 hr, 75% of the folates had been converted to the tetra- and pentaglutamate forms. After 24 hr, presumably a steady-state relationship was reached and over 90% of the labeled folates were in the penta- and hexaglutamyl forms, with the remainder consisting of tetra- and heptaglutamates.

The polyglutamate derivatives of pteroylglutamic acid have for some time been known to be quantitatively important (Pfiffner *et al.,* 1946; Hutchings *et al.,* 1948; Noronha and Silverman, 1962; Noronha and Aboobaker, 1963; Sirotnak *et al.,* 1963; Bird *et al.,* 1965; Schertel *et al.,* 1965), and a number of workers (Wright, 1956; Blakley, 1957; Guest and Jones, 1960; Kisliuk and Woods, 1960; Large and Quayle, 1963; Wang *et al.,* 1967; Whitfield and Weissbach, 1968; Burton *et al.,* 1969; Kozloff *et al.,* 1970) have been able to demonstrate the functional importance of some of these derivatives. Since methods have been developed for the solid phase synthesis of poly-γ-glutamates (Krumdieck and Baugh, 1969), it has become possible to consider more exact determination of poly-γ-glutamate chain lengths and their functions in different tissues.

Most workers who have attempted to determine the natural polyglutamates (Shin *et al.,* 1972; Osborne-White and Smith, 1973; Thenen *et al.,* 1973) have undertaken to identify them with regard to both the poly-γ-glutamyl chain length and the one-carbon substituents. However, due to the instability of the folates, their coexistence with many enzymes capable of modifying them, and the theoretically large number of possible molecular modifications, separation and identification of individual molecules become a very complex problem. Quantitative conversion of pteroylglutamyl derivatives to their corresponding *p*-aminobenzoylglutamate derivatives, which are relatively stable molecules, provides a solution to these problems.

Two approaches have been employed for cleavage of the C^9–N^{10} bond of pteroyl glutamates to yield the corresponding *p*-aminobenzoyl glutamates. One of these, in which the molecules are cleaved by an oxidative procedure, was used by Houlihan and Scott (1972) and Brown *et al.* (1974) in studies of mammalian folates. The other approach, which involves reductive cleavage of the molecules, has recently been developed in this laboratory (Baugh *et al.,* 1974).

The method employed by Houlihan and Scott (1972) makes use of heating and autoclaving to stop conjugase activity, procedures which may well result in alteration of the folate molecules. Additionally, they do not document any control experiments to establish the validity of the results obtained by the procedure used. On the other hand, the method employed in the previous paper (Baugh *et al.,* 1974) used 5% trichloroacetic acid in 6 M urea at low temperatures to stop conjugase activity, precipitate the proteins, and leave the folates in solution. It is also established by the appropriate control experiments that the procedure used for cleavage of the C^9–N^{10} bond does in fact cleave only that bond, leaving the γ-peptide linkages intact.

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TABLE I: Incorporation of Injected Pteroyl[^{14}C]glutamic Acid into Rat Liver.

Time after injection	cpm in liver	% of amount injected[a]
1 hr	154,000	3.4
3 hr	124,000	2.7
6 hr	194,000	4.3
24 hr	182,000	4.0
7 days	132,000	2.9
14 days	87,000	1.9
28 days	22,000	0.5

[a] Amount of Pte-G* injected was 4.5×10^6 cpm in all experiments.

Materials and Methods

Male Sprague-Dawley rats weighing between 200 and 250 g were used for all experiments. Pteroyl[U-^{14}C]glutamic acid (Pte-G*,[1] specific activity 5.12 Ci/mol) was synthesized in this laboratory by the solid phase method of Krumdieck and Baugh (1969), and was repurified by DEAE-cellulose Cl^- column chromatography prior to use. The *p*-aminobenzoic acid was obtained from commercial sources and polyglutamate derivatives of it synthesized by the solid phase procedure (Baugh *et al.*, 1974; Kisliuk *et al.*, 1974). Other chemicals were reagent grade obtained from regular commercial sources.

Preparation of Rats. Rats, fed a nutritionally complete diet, were given 4.5×10^6 cpm Pte-G* (175 μg) by intraperitoneal injection. At fixed time intervals after injection, 1 hr, 3 hr, 6 hr, 24 hr, 7 days, 14 days, and 28 days, rats were killed and the distribution of ^{14}C-labeled derivatives of the injected Pte-G* in the liver was determined.

Isolation and Cleavage of Rat Liver Folates. At the above-mentioned times after injection, rats were anesthetized with chloroform and the liver was removed as quickly as possible. The liver was washed thoroughly with ice-cold 0.9% NaCl, placed in 5% trichloroacetic acid in 6 M urea to a final volume of 80 ml, and homogenized in an ice bath for 5 min at maximum speed with a Virtis homogenizer. The homogenate was then centrifuged for 397,000*g*-min at 4°. The supernatant, which contained over 90% of the radioactivity from the liver homogenate, was decanted and the precipitate discarded. From this point the reductive cleavage and the analytical chromatography reported in the previous paper (Baugh *et al.*, 1974) were followed without modification.

Radioactivity Measurements. All samples were counted for radioactivity in 10 ml of 4% Cabosil in Multisol (Isolab, Inc.) in a Beckman LS-355 liquid scintillation system. Efficiency of counting was of the same order for all samples.

Results

Table I shows total radioactivity in the liver homogenate at the times examined. Maximum uptake of the labeled folate, which was 4.0–4.5% of the injected dose, can be seen at 6–24 hr after injection. Following this peak there is a progressive decrease so that by 7 days the level of labeled folates had decreased to 67.4% of the maximum amount incorporated, by 14 days to 44.8%, and by 28 days to 11.6%.

[1] Abbreviation used is: Pte-G*, pteroyl[U-^{14}C]glutamic acid.

Figure 1, the elution pattern of the *p*-aminobenzoylglutamyl derivatives of the labeled hepatic folates at 6 hr after injection of Pte-G*, is typical of the elution profiles obtained in all experiments. This graph shows the clear definition between the *p*-aminobenzoyl glutamate derivatives of differing poly-γ-glutamate chain lengths.

Figure 2 is a composite graph of the elution patterns obtained at each of the time intervals examined, enabling a direct comparison of the distribution of the ^{14}C label at the different times. Table II shows the distribution of the label among the derivatives of different chain lengths as a function of the percentage of total label incorporated at a particular time.

Discussion

The experiments described have demonstrated the application of the methods of extraction, cleavage, and purification of natural folates described in the preceding article (Baugh, *et al.*, 1974) to the examination of the uptake and incorporation of Pte-G* into a mammalian tissue, in this case the rat liver.

For the determination of mammalian liver folate distribution of polyglutamates, this method has overcome the problems associated with previously reported methods. Most other methods employed in studying mammalian liver folates (Bird *et al.*, 1965; Shin *et al.*, 1972; Osborne-White and Smith, 1973; Thenen *et al.*, 1973; Corrocher *et al.*, 1972) have entailed the attempted separation of the different components in their natural forms with a variety of one-carbon substituents, possible differing states of reduction, and variable glutamyl chain lengths. These methods have required differential microbiological assays and use of crude conjugases (pteroylpolyglutamyl carboxypeptidases) in addition to chromatographic separation for the identification of the different components. The consequent amplification of the errors in each procedure makes interpretation of results difficult.

An approach similar to that used in the present study was employed by Houlihan and Scott (1972) in their study of rat liver folates. They cleaved the C^9-N^{10} bond of the molecules by an oxidative procedure to produce the *p*-aminobenzoyl glutamate derivatives. A number of differences can be observed between the results obtained when they examined liver polyglutamates 24 hr after the injection and our 24-hr experiment, notably their failure to observe any derivatives with a chain length longer than five glutamyl residues while we found 22% of the labeled folates at this time to contain six or seven glutamyl residues. They also found a significant amount of label in the monoglutamate form, while we observed none. These differences may indicate either that the oxidative procedure does result in some cleavage of the γ-peptide linkages as well as the C^9-N^{10} bond, or that heating and autoclaving the tissue is resulting in the modification of some of the folate molecules. Another noticeable difference in these two experiments is that Houlihan and Scott recovered some 50% of the injected dose in the liver at 24 hr, while we recovered only 4%. This is a reflection of the different specific activities of pteroylglutamic acid injected. In fact, the absolute amount of pteroylglutamic acid incorporated into the livers of our rats at 24 hr was some 11.5 times that incorporated into the livers of their rats (7.00 μg as compared to 0.61 μg).

A similar approach to the identification of pteroyl polyglutamate forms in the liver and other tissues of the monkey has been reported (Brown *et al.*, 1974). These workers also

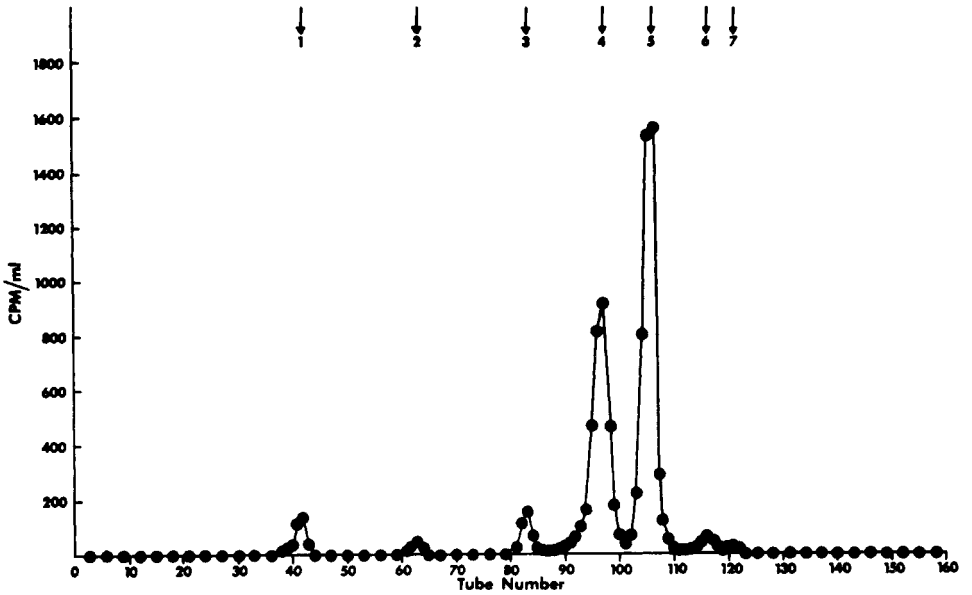

FIGURE 1: Elution profile of *p*-aminobenzoyl glutamate derivatives of ^{14}C-labeled pteroyl glutamates present in rat liver at 6 hr after injection of Pte-G*. The numbers under the arrows indicate the number of glutamyl residues in the eluted peak. Sample volumes were 12.5 ml.

employed techniques of boiling (5–10 min) and autoclaving (115°, 10–15 min) and an oxidative ($KMnO_4$–H_2O_2) procedure for the cleavage of the C^9–N^{10} bond. Obviously, differences between their results in the monkey and ours in the rat could also be due to species differences.

The uptake of an injected dose of Pte-G* into the liver of a healthy rat fed a nutritionally complete diet has been shown in this paper to reach a maximum at 6–24 hr (Table I), when some 4.0–4.5% of the injected dose was recovered from the liver. Following this peak of uptake, it was possible to observe a progressive decrease in the concentration of labeled derivatives, such that after 7 days the level decreased to 67.4% of the maximum, by 14 days to 44.8%, and by 28 days to 11.6%.

As indicated in the Results section of this paper, Table I shows that the level of labeled folates was actually higher at 1 hr than at 3 hr. This may be due to the higher level of label in the blood perfusing the liver at that time. Although the liver was thoroughly washed with ice-cold 0.9% saline after removal and before homogenization, it was not possible to remove all blood from the organ. Presumably at 1 hr after an intraperitoneal injection there would be a high level of the labeled folate in the portal blood. It may be that much of the label detected at this time was actually in the blood and not in the hepatic cells.

After absorption of pteroylglutamic acid into the rat liver cells there was a rapid addition of glutamyl residues, so that within 3 hr after injection of a dose of Pte-G* into a rat, 75% of it had been converted into the tetra- and pentaglutamyl derivatives. These results indicate that the monoglutamate derivatives of pteroylglutamic acid are not natural coenzymatic forms in the rat liver. This would also appear to be true for the di- and triglutamyl derivatives, which probably occur only as intermediates between the absorbed monoglutamates and the metabolically active polyglutamates or for transport across cellular, or intracellular, membranes.

After the initial rapid conversion to derivatives with polyglutamyl chains containing more than three glutamyl residues, there follows a slower process during which there is further addition of glutamyl moieties until, after 7 days, over 90% of the label can be detected in the penta- and hexaglutamates. These derivatives appear to be the predominant folates in the rat liver at equilibrium, with the remainder of the label being located in the tetra- and heptaglutamates.

Most of the label which becomes incorporated into tetraglutamyl derivatives during the first 6 hr after injection of

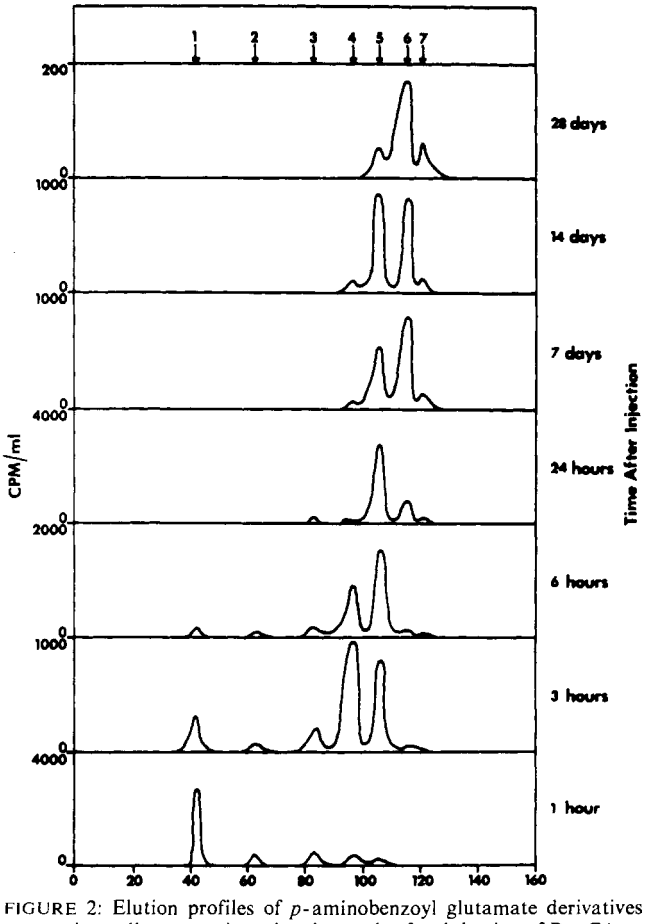

FIGURE 2: Elution profiles of *p*-aminobenzoyl glutamate derivatives present in rat liver at various time intervals after injection of Pte-G*.

TABLE II: Incorporation of Pte-G* into Rat Liver Folates, Expressed as Per Cent of ^{14}C Label in Each $pABG_n$ Derivative at Different Time Intervals.

Time	pABG	$pABG_2$	$pABG_3$	$pABG_4$	$pABG_5$	$pABG_6$	$pABG_7$
1 hr	67.7	9.2	10.6	10.4	2.0	0.0	0.0
3 hr	13.6	0.8	8.9	10.5	35.1	1.1	0.0
6 hr	5.0	1.1	4.6	36.5	50.5	1.6	0.7
24 hr	0.0	0.0	0.5	1.7	76.7	20.7	0.4
7 days	0.0	0.0	0.0	2.1	35.8	56.7	5.4
14 days	0.0	0.0	0.0	4.3	49.2	41.3	5.2
28 days	0.0	0.0	0.0	0.0	20.2	53.6	26.2

Pte-G* appears to be in transit to the higher forms. However, from 24 hr to 14 days it was consistently possible to find a small percentage of the label in the tetraglutamates. The preceding article (Baugh *et al.*, 1974) showed the tetraglutamates to be the major components of *Lactobacillus casei, Streptococcus faecium,* and a methotrexate-resistant mutant of *Streptococcus faecium.* These results would seem to reflect the differences in the metabolic functions of bacterial cells and mammalian liver cells, which lead to different requirements for folate coenzymes. Perhaps the major difference between the bacterial cells and the rat liver cells is that most of the bacterial cells are in the log phase of growth and therefore continually producing new DNA for cell division, whereas there is no significant turnover of normal rat liver cells.

Since after 24 hr the hexa- and pentaglutamates together comprise over 90% of the total radioactive folates, it would appear that these forms are involved in the major pathways of one-carbon metabolism in rat liver. The results showed that at equilibrium there is some variation in the relative levels of the penta- and hexaglutamates, although their total combined level remains relatively constant. This may indicate that there is a certain amount of interconversion between these two forms according to the metabolic requirements of the animal. As the levels of labeled folates in the liver become depleted, after 28 days, one can see that the percentage of the label found in these derivatives decreases, disappearing from the pteroyl pentaglutamates more rapidly than from the hexaglutamyl form.

Maximum incorporation of radioactivity into the heptaglutamyl derivatives was seen at 7 days. The relative level of labeled heptaglutamates was the same at both 7 and 14 days, but by 28 days it had greatly increased so that this fraction comprised some 26.2% of the total label. This relative increase in label observed in the heptaglutamates as the labeled folates became depleted reflects the much slower rate of turnover of this fraction. While the experiments described in this report do not allow one to come to any definite conclusions as to the reason for the much slower rate of turnover of the heptaglutamyl fraction, one could speculate that this reflects a storage function for this form.

In conclusion, the results described appear to indicate that an injected dose of Pte-G* reaches an equilibrium with the hepatic folate pools within 7 days. This is compatible with the findings of Corrocher *et al.* (1972) in their study of guinea pig liver folates, but disagrees with the figure of 24 hr stated by Shin and coworkers (1972) and Houlihan and Scott (1972) for rat liver folates.

One must exercise a certain amount of caution in the interpretation of results from experiments in which one examines the incorporation of a single injection of radioactive folates into the nonlabeled folate pool of a tissue. If radioactivity is the only analytical tool employed, those nonlabeled forms which may have half-lives of far longer duration than the time course under study, or which may not be in equilibrium with the circulating folates, will not be detected. Nevertheless, it is felt that the data reported here provide a meaningful insight into the patterns of uptake and metabolism of folic acid in rat liver and of the distribution of pteroyl polyglutamates in that tissue.

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25-Hydroxyvitamin D_3-1-Hydroxylase. Inhibition *in Vitro* by Rat and Pig Tissues†

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ABSTRACT: Although 25-hydroxyvitamin D_3-1-hydroxylase can easily be demonstrated *in vitro* with chick kidney preparations, attempts to demonstrate this activity *in vitro* with rat kidney homogenates have been unsuccessful, despite the fact that nephrectomized rats cannot produce 1,25-dihydroxyvitamin D_3. The failure of the rat preparation to carry out this hydroxylation has now been shown to be due to the presence of a heat-labile, highly potent inhibitor of the reaction. This inhibitor was found in all kidney cell fractions, but the most potent appeared to be the microsomes which released the inhibitor during incubation. A similar inhibitor was found in intestine and blood serum, both being potent sources. Pig kidney tissue also contained such an inhibitor.

The metabolism of vitamin D_3 to its more biologically active hydroxylated derivatives has been reviewed extensively (Olson and DeLuca, 1973; Omdahl and DeLuca, 1973; Wasserman and Taylor, 1972). 25-Hydroxyvitamin D_3 (25-OH-D_3)[1] is known to be synthesized mainly in the liver (Horsting and DeLuca, 1969) and represents the major circulating metabolite of the vitamin (Ponchon and DeLuca, 1969; DeLuca, 1969; Mawer *et al.*, 1969).

The second hydroxylation step, which has been shown by experiments with nephrectomized rats to be carried out only in the kidney (Fraser and Kodicek, 1970; Gray *et al.*, 1971; Omdahl *et al.*, 1972), occurs either at the 1 or the 24 position of the 25-OH-D_3 molecule (Holick *et al.*, 1971a,b, 1972) depending on the vitamin D, calcium, and phosphorus status of the animal. 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the most active form of vitamin D_3 known in both induction of intestinal calcium transport and bone mobilization (Holick *et al.*, 1971a; Haussler *et al.*, 1971; Omdahl *et al.*, 1971), is produced by hyposphosphotemic and hypocalcemic animals, whereas normal calcemia, hypercalcemia, or hyperphosphatemia leads to the production of 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) (Boyle *et al.*, 1971; Tanaka and DeLuca, 1973).

In vitro hydroxylation of 25-OH-D_3 in the 1 position (Fraser and Kodicek, 1970; Gray *et al.*, 1971, 1972; Norman *et al.*, 1971) and in the 24 position (Omdahl *et al.*, 1972; Knutson and DeLuca, 1974) has been demonstrated using both homogenates and isolated mitochondria from kidneys of chicks fed the appropriate diet. Midgett *et al.* (1973) have reported the presence of a compound which co-chromatographs with 1,25-$(OH)_2D_3$ after incubation of 25-OH-D_3 with human, rat, and dog kidney homogenates; however, all attempts made in this laboratory to establish a rat kidney system which will synthesize either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ *in vitro* have been unsuccessful.

This paper reports the presence of a factor in rat kidney homogenates which inhibits the formation of 1,25-$(OH)_2D_3$ from 25-OH-D_3 by homogenates and isolated mitochondria prepared from kidneys of vitamin D deficient chicks. As it seems possible that this factor may represent a regulator of 1,25-$(OH)_2D_3$ synthesis, some of its properties are reported.

Materials and Methods

Animals. One-day-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, Wis.) were maintained on a vitamin D deficient purified diet (Omdahl *et al.*, 1971) for 4–6 weeks before use.

Male albino rats (Holtzman Co., Madison, Wis.) were housed in hanging wire cages and fed a low vitamin D stock diet (Steenbock, 1923) or a vitamin D deficient diet containing 0.47% calcium and 0.3% phosphorus (Suda *et al.*, 1970). Variation in the calcium content of the diet was balanced by appropriate changes in the sugar content. When required, vitamin D_3 (1 or 2 IU daily) was given orally in 0.1–0.2 ml of cottonseed–soybean oil (Wesson Co., Fullerton, Calif.). Rats were allowed food and water *ad libitum* for 5–8 weeks before being used in experiments.

Pig kidneys were taken from two animals of mixed breed weighing 250–300 lb which had been fed stock rations, supplemented with enough vitamin D_2 to supply each pig with 500,000 IU daily for 28 days (Suda *et al.*, 1969).

For *in vivo* experiments rats were injected intrajugularly (under ether anethesia) with 650 pmol of 25-OH-[26,27-^{3}H]D_3 (1.2 Ci/mmol) in 0.05 mol of 95% ethanol 24 hr be-

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[1] Abbreviations used are: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3.