Hepatic Parenchymal Cell Glutamylation of Methotrexate Studied in Monolayer Culture

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ABSTRACT

A detailed analysis of the γ-glutamylation of methotrexate has been conducted in primary cultures of rat hepatic parenchymal cells in monolayer culture. The rates of glutamylation are concentration dependent and saturable when measured over a 6-h period at concentrations between 2 and 50 μM. The removal of folate and inclusion of dexamethasone, and tocopherol enhance glutamylation. Omission of methionine from the medium increases glutamylation, whereas an increase in methionine represses the reaction. During the 6-h period of syntheses, methotrexate diglutamate is the primary product, whereas the di- and triglutamates are the major cellular species when the incubation is extended to 24 h. Lower extracellular methotrexate concentrations result in the formation of relatively greater amounts of longer chain length derivatives.

The accumulation of methotrexate polyglutamates at steady state is saturable and occurs by 24 h. The predominant species contain two to four glutamate residues, and the distribution depends upon the culture conditions and the extracellular methotrexate concentration. The turnover of cellular polyglutamates at saturation occurs at 30 to 40% of the total cell pool over a 6-h period. Placement of hepatocytes with saturating levels of methotrexate polyglutamates in medium lacking methotrexate results in the slow loss of all derivatives, and the rate of loss is inversely related to polyglutamate chain length.

Following a pulse dose of methotrexate, hepatocytes continue to increase the chain length of the cellular pool of polyglutamates, and this process is impaired by addition of folic acid to the medium. In the pulse experiments as in the longer term incubations, the primary species are the di- and triglutamates. The results demonstrate limited capacity of the hepatocytes to glutamylate longer chain length polyglutamate derivatives.

INTRODUCTION

The interaction of the antifolate, methotrexate (4-amino-10-methylpteroylglutamic acid), with liver is an important parameter in the overall activity of this drug. Methotrexate is transported via active, carrier-mediated transport systems into the hepatocytes (1-4) and secreted into bile against a concentration gradient (5). While in rat hepatocytes, methotrexate can be bound and this process is impaired by addition of folinic acid to the medium. The accumulation of methotrexate polyglutamates is of great interest with regard to the hepatotoxicity which can accompany methotrexate administration, since these are retained by hepatocytes for longer periods of time than methotrexate (2, 6-10).

Definitive descriptions and complete mechanistic assessments of all the aspects (transport, efflux and biliary secretion, binding to dihydrofolate reductase, intracellular sequestration, polyglutamylation, hydroxylation, and possibly others) of the handling of methotrexate by the liver are not available, but initial studies have been made in all these areas. The seminal observation of the glutamylation of methotrexate was made in rat liver by Baugh et al. (10), followed by a more detailed evaluation of these processes in vivo by other investigators (11, 12).

The early studies were extended by the use of suspensions (2, 3, 6) and monolayer (4, 7, 9, 13, 14) cultures of hepatocytes. The results indicate that methotrexate is γ-glutamylated to forms containing a total of two to five glutamate residues and that these are retained by the hepatic parenchymal cells more avidly than is methotrexate (2, 6, 7-9). The accumulation of methotrexate polyglutamates at steady state was found to be saturable with respect to the extracellular methotrexate concentration (8).

Because of the importance of this reaction in contributing to hepatotoxicity and the potential for using methotrexate to clarify the nature of γ-glutamylation in hepatic tissue, a more detailed analysis of the metabolism of methotrexate to polyglutamates in cultured hepatocytes is presented here.

MATERIALS AND METHODS

Materials. Swim’s Medium S-77, folate-free Swim’s Medium S-77, L-15 medium, fetal bovine serum, and horse serum were obtained from Grand Island Biological Co. (Grand Island, NY). Methotrexate and [3,5,7-3H]methotrexate were supplied by Lederle Laboratories (Pearl River, NY) and purchased from Amersham/Searle Corp. (Arlington Heights, IL), respectively. Both compounds were purified by DEAE-cellulose column chromatography (15) prior to use. The concentration of methotrexate was determined by the extinction coefficient at 302 nm (16). The final specific activity of [3H]methotrexate in these experiments was in the range of 1 to 5 × 106 dpm/nmol. Methotrexate polyglutamate standards (Glu2 to Glu5) were kindly provided by the National Cancer Institute. DEAE-cellulose was purchased from Schleicher and Schuell (Keene, NH). Collagenase (type I), dexamethasone, insulin, and α-tocopherol were purchased from Sigma Chemical Co. 7-Hydroxymethotrexate was kindly provided by Dr. J. J. McGuire of Yale University, and 7-hydroxymethotrexate polyglutamates, by Dr. P. Newton and Dr. R. L. Blakley of St. Jude’s Children’s Hospital.

Animal Maintenance and Cell Culture. Adult male Lewis rats were supplied by Charles River Breeding. The animals were kept on a 12-h light-dark cycle and fed Wayne Lab Blox F6 and water ad libitum. The rats were used when their body weight was 200 to 275 g. The hepatocytes were isolated as previously described (4). The cells were plated at a density of 2.5 × 10⁴ cells/ml of L-15 medium with insulin (10 milliunits/ml) and 15% fetal bovine serum on a 60-mm Falcon dish. Attachment at 37°C occurred within 3 to 4 h, and the medium was then changed to

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2 To whom requests for reprints should be addressed.

3 J. Galivan, unpublished results.
Glutamylation of methotrexate by cultured hepatocyte and hepato
toma cells. The uptake of methotrexate and its conversion to polyglu
tamate derivatives in monolayer-cultured hepatocytes were conducted
as described previously (7–9). After the cells were cultured for 24 h,
[^3H]methotrexate was added in fresh medium for metabolic studies.
At the end of the incubation, the plates were cooled in the ice bath
and washed 4 times with ice-cold phosphate-buffered saline. The cells
were removed from the plates by scraping with a rubber policeman into
two washes of 1 ml of NaOH or 1 ml of distilled water. The cells were
extracted with NaOH to measure the total cellular methotrexate and
polyglutamate pool and to measure cellular protein (8). The distribu
tion of polyglutamate derivatives was evaluated by HPLC (17). The rates
of glutamylation are expressed in nmol/g of cellular protein and refer
to either the conversion of methotrexate into the polyglutamate pool or
the number of glutamate residues added (glutamylation).

Formation of 7-hydroxymethotrexate and the diglutamate, 7-OH-4-
NH2-10-CH3PteGlu, was also observed in the hepatocytes when assay
ned by the procedure of Newton and Blakely (18). In all results reported
here, the amount of 4-NH2-10-CH3PteGlu was corrected for the amount
of radioactivity (25 to 45%) contributed by 4-NH2-7-OH-10-CH3PteGlu
which eluted with 4-NH2-10-CH3PteGlu on the HPLC system used to
separate methotrexate polyglutamates (17). This was done by subjecting
an identical sample to HPLC to resolve 7-hydroxy methotrexate polyglu
tamates and methotrexate diglutamate (18) and subtracting the contribu
tion made by 4-NH2-7-OH-10-CH3PteGlu. A separate report is being
prepared on the topic of hydroxylation of methotrexate in rat
hepatic cells.3

RESULTS

The rates of glutamylation of methotrexate by hepatocytes in
primary monolayer cultures as a function of extracellular con
centration are demonstrated in Fig. 1. Between 2 and 50 μM metho
trexate, there is an increase in glutamylation when the incuba
tion is conducted in Swim’s medium. The rate is enhanced
approximately 50% by the inclusion of insulin, dexamethasone,
and tocopherol, (Fig. 1B). Studies in which these components
are added independently showed that insulin alone exerted most
of this effect.3 The combination of the three agents was utilized
to stabilize methotrexate accumulation (4). A further enhance
ment in glutamylation occurs when folate is omitted from the
medium in addition to inclusion of the additives mentioned above
(Fig. 1C). Under these conditions, there is little difference in the
rate of glutamylation between 2 and 6 h when the data for 10
and 50 μM (as measured by the slope of the curves) extracellular methotrexate are compared. Some lag can be observed at the
low methotrexate concentrations between 0 and 2 h in Fig. 1C,
and this may be related to the relatively high Km of folypolyglu
tamate synthetase (EC 6.3.2.17) for methotrexate (19). A more
pronounced lag has been observed with hepatoma cells which
accumulate much lower cellular concentrations of methotrexate
(17).

The rate measurements shown for the various concentrations
were conducted after the cells had been in culture for 24 h. The
glutamylation of methotrexate at 50 μM was also examined in
cells which had been in culture for 48 h. The rates are lower at
48 h than at 24 h when folic acid is present (Fig. 1, A and B),
but in its absence, the rate is identical (Fig. 1C). These results
indicate that the capacity for glutamylation is relatively stable in
cultured hepatocytes, although it can be modified by the meta
bolic state of the cell. This evaluation, which is consistent with
earlier preliminary observations (8, 13), was conducted because of
the marked instability of the capacity of the cells in culture to
accumulate methotrexate (4).

The cell extracts from the experiment shown in Fig. 1 were
analyzed for the distribution of methotrexate polyglutamates (Fig.
2). Under the three culture conditions used, the diglutamate is
the predominant species over the 6-h incubation when methotrex
ate is present at 25 μM. Similar results were obtained at all
concentrations of methotrexate. Only modest amounts of the
triglutamate are formed, except in folate-lacking medium, and
under these conditions, some tetraglutamate was observed (Fig.
2C).

Polyglutamate formation during a 24-h incubation with methotrexate is a saturable process which results in the steady-state
formation of polyglutamate derivatives (8). The effects of culture
conditions and the extracellular methotrexate concentration on
the amounts of polyglutamates accumulated are shown in Fig.
3. The inclusion of insulin, dexamethasone, and tocopherol
causes the polyglutamate pool to be increased by more than 2-
fold (Fig. 3B), whereas the removal of folic acid from the supple
mented cells causes only a slight further increase in the pool
(Fig. 3C). Under all three culture conditions, the di- and triglu
matates are predominant when the extracellular methotrexate is
greater than 10 μM. At lower concentrations (2 and 5 μM), the
relative amounts of the tetraglutamate are greater and can
exceed the diglutamate (Fig. 3, B and C). Under all incubation
conditions, the amount of tetruglutamate decreases when the
methotrexate concentration is increased from 2 to 50 μM. Small
amounts of the pentaglutamate derivative are consistently ob
served.

Previous studies established the selective retention of the
methotrexate polyglutamate pool by hepatocytes (2, 6, 7–9). A
detailed evaluation of the individual polyglutamates is shown in
Table 1. Hepatocytes which had been incubated with methotrex
ate until steady state was reached (conditions described in Fig.
3C) were placed in medium lacking methotrexate. As demon
strated previously (2, 4), there is a relatively slow release of
methotrexate, indicating the presence of a nonexchangeable
pool. The remainder of the polyglutamates is retained in direct
relation with their chain length and the amounts of the tetra
and pentaglutamates actually increased over a 6-h period.

For comparison, analogous data with the H35 hepatoma cell
line are included. Each of the corresponding derivatives is lost
more rapidly by the H35 cells. The amounts of total polyglu
matates retained after 6 h are similar in hepatocytes and hepatoma
cells. This is due to the fact that the distribution of polyglutamates
in hepatoma cells favors the longer chain length polyglutamate
which offsets the greater retentive capacity of hepatocytes for
individual polyglutamates.

The turnover of the polyglutamate pools at steady state was
measured by incubating the hepatocytes for 24 h with unlabeled
methotrexate and replacing it with [3H]methotrexate. These ex
periments were conducted in Swim’s Medium S-77 (Fig. 4A) and
Fig. 1. The time- and concentration-dependent glutamylation of methotrexate in cultured hepatocytes. The glutamylation of methotrexate was measured after the hepatocytes had been in culture for 24 h. The medium was Swim's Medium S-77 (A); Swim's Medium S-77 with 10 milliunits of insulin per ml, 0.1 μM dexamethasone, and 6 μM tocopherol (B); and folate-free Swim's Medium S-77 (C) with the additives included in (B). The cultures were converted into these media after 3 h of attachment in L-15 medium with 15% fetal calf serum. [3H]Methotrexate was added in fresh medium at concentrations of 2 μM (●), 5 μM (□), 10 μM (●), 25 μM (△), and 50 μM (▲). The highest concentration (50 μM, ▲ ▲ ▲ ▲ ▲ ▲) was also examined after the cells had been in culture for 48 h. The glutamylation of methotrexate was measured as described in "Materials and Methods." Points, mean of triplicate measurements from a single hepatocyte preparation. The average standard error for all individual points is 7.1%. The variation in glutamylation among independent hepatocyte preparations (n = 5) was assessed by measuring the reaction at a methotrexate concentration of 25 μM. The standard error for glutamylation was 10.1% (A), 9.7% (B), and 8.2% (C).

Fig. 2. The synthesis of individual methotrexate polyglutamates by cultured hepatocytes. The experiment is similar to the experiment described in Chart 1, except that the individual polyglutamates were measured. Methotrexate was present at 25 μM in the medium. The culture conditions in A, B, and C correspond to identical conditions in Fig. 1. Points, mean of five independent hepatocyte preparations (each with triplicate samples); bars, SE.

Fig. 3. Steady state accumulation of methotrexate polyglutamates in cultured hepatocytes. The experiment is conducted in a manner identical to the conditions in Fig. 1 except that the incubation is for 24 h. PG refers to the total polyglutamate pool (Glu₂ through Glu₆). The results are the mean of triplicate samples of an individual experiment and average standard error for all points is 5.5%. The variation in glutamylation as a function of different liver cell isolations was evaluated at 25 μM methotrexate in 5 independent cell preparations. The S.E. for total polyglutamates was 11% (A), 14% (B) and 10% (C). In all cases the distribution among the individual polyglutamates was similar.

Folate-lacking Swim's medium with additives (Chart 4B). The rate of turnover under the latter condition is nearly twice that observed in unsupplemented medium. Approximately 40% of the polyglutamates' pool in Swim's Medium S-77 is turned over in 6 h and 32% in supplemented folate-free Swim's Medium S-77. Experiments utilizing [3H]methotrexate prior to and during the turnover phase of the experiment indicated no change in the amounts of the individual polyglutamates during the course of the turnover experiment.³ Previous studies with transformed hepatic cells have demon-
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Table 1
Retention of methotrexate polyglutamate by cultured hepatocytes

Hepatocytes were incubated with 20 μM methotrexate for 24 h as described in Fig. 3. The medium was then replaced with fresh medium lacking methotrexate, and the cellular contents were measured as a function of time. An analogous experiment was conducted with H35 cells which had been incubated for 24 h with 10 μM methotrexate in folate-lacking Swim’s Medium S-77 with 10 milliunits of insulin per ml. The data were derived as described in Fig. 3.

<table>
<thead>
<tr>
<th>Glutamate content (nmol/g)</th>
<th>H</th>
<th>Glu2</th>
<th>Glu3</th>
<th>Glu4</th>
<th>Glu5</th>
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<td>14.5</td>
</tr>
</tbody>
</table>

| **Hepatoma cells**        | h |      |      |      |      |
| 0.5                       | 25.5 | 17.9 | 54.4 | 141.2 | 85.2 |
| 1.0                       | 8 | 10.4 | 46 | 120 | 76 |
| 2.0                       | 4.1 | 4.5 | 31 | 107 | 65.6 |
| 4.0                       | 3.2 | 2.2 | 19.1 | 92.5 | 67.3 |
| 6.0                       | 3.6 | 3.1 | 10.4 | 62 | 62 |

Fig. 4. Turnover of methotrexate polyglutamates (PG) in cultured hepatocytes. Hepatocytes which had been in culture for 24 h in Swim’s Medium S-77 (A) or folate-lacking Swim’s Medium S-77 with 10 milliunits of insulin per ml, 0.1 μM dexamethasone, and 6 μM tocopherol were exposed to a 3-h pulse of 20 μM [3H]methotrexate. At that time (arrow), the medium was replaced with fresh medium that lacked (—) or contained 50 μM folinic acid (——). Points were analyzed as in Fig. 3.

**DISCUSSION**

Previous studies by Foo and Shane (21) have shown that the folypolyglutamate synthesis in Chinese hamster ovary cells is influenced by methionine. Analogous experiments are reported here with hepatocytes and H35 hepatoma cells (Table 2). The presence of methionine caused a marked reduction in glutamylation in hepatocytes which was independent of the presence of folinic acid in the medium. In contrast, methionine had no effect on glutamylation in H35 cells in folate-repleted medium, but the absence of methionine in folate-restricted medium caused a marked reduction in glutamylation. The reason for the opposing effects of methionine on glutamylation in hepatocytes and hepatoma cells is not clear at this time, but the results are probably related to the complex metabolic differences between normal and transformed hepatocytes (22).
process in somewhat greater detail. The former authors established that higher doses of methotrexate resulted in more extensive glutamylation, and the latter showed that as many as three glutamate residues could be added to the parent drug. In addition, Sirotnak and coworkers (11) showed that relatively high levels of unmetabolized methotrexate were found in liver.

The formation of methotrexate polyglutamates in cultured rat hepatocytes was first established by this laboratory (13) concurrently with the studies of Goldman and coworkers (6). Hepatocytes were among the first culture systems which were utilized to demonstrate that methotrexate polyglutamates are selectively retained by cells relative to methotrexate (2, 6, 7). Some preliminary studies have been made on the characteristics of methotrexate polyglutamate formation in cultured hepatocytes, although these studies have not been very extensive (2, 6, 8). Furthermore, none of the previous studies was concerned with the contribution of 7-hydroxymethotrexate. The present study was conducted to demonstrate a detailed analysis of the formation of the polyglutamate derivatives of methotrexate. By the use of the HPLC systems described here, the contribution of the 7-hydroxy derivatives is eliminated.

The rate of synthesis and accumulation of methotrexate polyglutamates in hepatocytes are saturable and altered by the culture conditions. As with H35 hepatoma cells (17, 23), the omission of folates and inclusion of insulin increase the rate of glutamylation of methotrexate, although the effect is less dramatic. The absolute values for glutamylation under optimal conditions (folate lacking, insulin containing) are approximately one-fifth that observed with hepatoma cells. The hepatocytes differ further from hepatoma cells in that the longer chain length polyglutamates (Glu₄ and Glu₅) are far less extensively generated. These studies establish that, regardless of the conditions, the diglutamate is the chief product over 6 h and is a major component at 24 h except at low methotrexate concentrations. In hepatoma cells, relatively large amounts of the Glu₃ and Glu₄ conjugates are seen within 4 to 6 h (17, 19), and the diglutamate is always a minor component. These differences may reflect differences in folypolyglutamate synthetases from the two sources or in the regulation of polyglutamate synthesis and cleavage. Further studies are needed to clarify these points.

Both hepatocytes and hepatoma cells are similar in that a lower extracellular concentration leads to the formation of relatively longer chain length polyglutamates. Similar results have been found with regard to folypolyglutamate synthesis in rat hepatoma cells in vitro (24) and with partially purified folypolyglutamate synthetase from rat (19) and mouse (25) liver. This characteristic of polyglutamate formation may be a consistent feature of folypolyglutamate synthetase from varied sources.

The effect of folate omission on the rate of polyglutamate formation and steady-state accumulation differs. Removing folates from the medium causes an increase in the rate of glutamylation but very little effect on accumulation. The reasons for this are not yet understood, but it is not unreasonable that different factors control the rate of the reaction and steady-state accumulation. Analysis of the folate pools is required to approach an understanding of this apparent paradox. The more rapid rates of glutamylation observed in the absence of folates may be explained by the observations of Priest and coworkers (23). In these studies, it was shown that folate omission causes a marked reduction in the amount of shorter chain length folypolyglutamates, and the predominant chain length species are lengthened. Since the shorter chain length species would be most likely to compete with methotrexate for folypolyglutamate synthesis (19), a reduction in their concentration would favor the glutamylation of methotrexate.

A comparison of activity of folypolyglutamate synthetase activity extracted from rat liver and the capacity of rat hepatocytes to convert methotrexate to polyglutamates indicates that the enzyme in extracts may have sufficient catalytic capacity to accommodate this activity (19). Direct comparisons are not straightforward because (a) liver extracts include material from cells other than hepatocytes and (b) the enzyme is assayed at pH 9.0, whereas the cellular contents are closer to neutral pH. However, at substrate saturation (100 µM or greater), the enzyme has an activity slightly in excess of 300 nmol/g/h in crude extracts (26). The maximal rates observed with cultured hepatocytes are approximately 15 nmol/g/h. Further experimentation with partially purified enzyme showed that the di- and triglutamates were primary products with methotrexate as the substrate (26). This is also true of hepatocytes, especially with shorter incubations and higher methotrexate concentrations (Figs. 2 and 3), but the intact cells can produce measurable amounts of tetra- and pentaglutamates. These data suggest that the enzyme from rat liver has a relatively low affinity for the tri- and tetraglutamate derivatives of methotrexate. This conclusion is in agreement with analogous studies of McGuire and coworkers (26) with isolated folypolyglutamate synthetase. The reduced affinity for higher polyglutamates may be in part responsible for the lowered rate of glutamylation in hepatocytes when compared to hepatoma cells.

The interaction of methotrexate with hepatic tissue of clinical importance is chiefly related to the toxicity of the drug upon long-term exposure (27–29). The reason for the toxicity is not obvious, because the liver consists of nondividing hepatic parenchymal cells which are in the G₀ state (30). Thus, very little de novo synthesis of thymidylate occurs, resulting in a minimal production of dihydrofolate. Hence, the inhibition of the target enzyme, dihydrofolate reductase, may play a limited role in methotrexate-induced hepatotoxicity. Recent studies have shown the possibility of alternate enzymic sites for methotrexate (28), and these may be involved in methotrexate hepatotoxicity. It has been shown that methotrexate does compromise hepatic folate levels in humans (31). However, neither the mechanism nor the consequences of this are understood.

Two experiments in this study demonstrate possibilities for reducing the glutamylation of methotrexate in hepatocytes and, hence, its intracellular concentration. The addition of folic acid following a 3-h pulse of methotrexate prevents further glutamylation (Fig. 5) despite the fact that the hepatocytes contain very high concentrations of unmetabolized methotrexate (250 to 400 nmol/g) under these conditions (4). Methionine has been shown to cause a reduction in glutamylation in hepatocytes which may be somewhat selective, since it either enhanced or had no effect on glutamylation in hepatoma cells. This may be especially interesting, since data have been presented which predict that methotrexate toxicity to liver is caused by impairing methylation processes (29).

The present study yields a detailed description of the metabolic capabilities of hepatic parenchymal cells with regard to the glutamylation of methotrexate. Further experimentation is under
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way to evaluate the formation of 7-hydroxymethotrexate in hepatocytes (32) and the effects of the parent drug, methotrexate, and its metabolites on hepatocellular folate pools. It is the ultimate purpose that a greater understanding of methotrexate-induced hepatotoxicity will be achieved.

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