Synthesis of folate polyglutamates in human cells

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Summary
1. Synthesis of polyglutamate derivatives from radioactively labelled folic acid, folinic acid and methotrexate has been studied in human phytohaemagglutinin-transformed lymphocytes.

2. With labelled folic acid as the precursor, approximately 20% of the labelled cell folate was found in each of the mono-, tetra-, penta- and hexa-glutamate peaks after 72 h of incubation. At earlier times, the amounts of labelled folate monoglutamate and lower folate polyglutamates were proportionately greater and the amount of labelled higher folate polyglutamates was lower. After only 4 h incubation over 80% of the intracellular labelled folate was still in the monoglutamate form.

3. With labelled folinic acid used as precursor, approximately 30% of labelled cell folate after 72 h incubation was in the form of folate pentaglutamate, with tri- and tetra-glutamate forming the other major peaks. The speed of formation of polyglutamate derivatives was substantially more rapid than from folic acid.

4. Methotrexate was found to decrease considerably the amount of folate polyglutamate formation from labelled folic acid in lymphocytes but nevertheless some di-, tri- and traces of tetra-glutamate were formed. On the other hand, methotrexate had no effect on folate polyglutamate formation with labelled folinic acid used as the precursor.

5. Polyglutamate derivatives of labelled methotrexate were formed by human lymphocytes with mean amounts of 4.5% of di-, 1.5% of tri-, 1.0% of tetra- and 0.7% of penta-glutamate derivatives being formed over the period 48–72 h of culture.

6. Pentaglutamate derivatives probably constitute the largest group of intracellular folates in human cells but a complex mixture exists.

7. The enzyme synthesizing polyglutamate derivatives of folate in human cells prefers a reduced folate as substrate but the requirement for a reduced form is not absolute. The nature of the reduced folate is uncertain but it is suggested on the basis of previous work that tetrahydrofolate rather than methylhydrofolate or any other reduced folate monoglutamate is the preferred substrate.

Key words: folate, folate polyglutamates, folinic acid, lymphocytes, methotrexate.

Introduction
There is now strong evidence that the natural coenzymes of folate in mammalian cells are the polyglutamate derivatives of the reduced folate compounds. Folate polyglutamates form almost all the intracellular folates in mammalian cells, including those in resting and rapidly proliferating phases (Lavoie, Tripp, Parsa & Hoffbrand, 1975). Direct biochemical studies in which folate polyglutamate and folate monoglutamate derivatives have been compared as coenzymes in folate-mediated reactions in mammalian systems have invariably shown that the polyglutamate derivative is more active (Blakley, 1957; Coward, Parameswaran, Cashmore & Bertino, 1974; Coward, Chello, Cashmore, Parameswaran, De Angelis & Bertino, 1975; Cheng & Stokstad, 1975). Moreover, a strain of Chinese hamster cell
which is able to incorporate folate monoglutamate but unable to synthesize folate polyglutamates, requires extra thymidine, adenosine and glycine for growth, compounds whose synthesis requires folate-mediated reactions (McBurney & Whitmore, 1974a, b).

In view of all these observations, the mechanism of synthesis of the folate polyglutamate derivatives assumes considerable interest. Studies of synthesis of polyglutamate derivatives of folate from labelled folic acid have now been carried out in vivo in the guinea-pig, rat and monkey (Corrocher, Bhuyan & Hoffbrand, 1972; Houlihan & Scott, 1972; Shin, Williams & Stokstad, 1972; Shin, Buehring & Stokstad, 1974; Brown, Davidson & Scott, 1974a,b; Leslie & Baugh, 1974). Folate polyglutamate synthesis has also been studied in vitro with a semi-purified cell extract from Neurospora crassa (Sakami, Ritari, Black & Rzepka, 1973; Ritari, Sakami, Black & Rzepka, 1975) and from rat liver (Spronk, 1973) and in a broken cell extract of sheep liver (Gawthorne & Smith, 1974).

In the only studies of folate polyglutamate synthesis in human cells so far reported, Lavoie, Tripp & Hoffbrand (1974) found that human phytohaemagglutinin-stimulated lymphocytes synthesize labelled folate polyglutamate derivatives from [3H]folic acid in vitro over a 72 h period and that the synthesis with labelled folic acid used as precursor was not impaired by vitamin B12 deficiency. Indirect evidence in these studies suggested that methyltetrahydrofolate was not the direct substrate for the folate polyglutamate-synthesizing enzyme but that the methyl group was removed before polyglutamate synthesis occurred, a 'demethylation' which presumably requires vitamin B12.

In the present studies, we report the size of polyglutamates formed in human cells from tritium-labelled folic acid (pteroylglutamic acid) and tritium-labelled folinic acid (5-formyltetrahydrofolic acid), the time-course of synthesis of labelled folate polyglutamates in human cells and effects of methotrexate on this process. In addition, synthesis of labelled polyglutamates from tritium-labelled methotrexate itself has been found to occur in human cells. An abstract of part of this work has been published (Lavoie & Hoffbrand, 1974).

**Materials and methods**

Lymphocytes were obtained from venous blood collected from normal adult volunteers, separated by the Triosil-Ficoll method (Boyum, 1968) and cultured with phytohaemagglutinin over a 72 h period as described by Das & Hoffbrand (1970). Labelled precursors were added, 2 μCi in 20 μl of sodium chloride (150 mmol/l) to each of the 3 ml culture bottles containing 3 × 10⁶ cells. Trito tiated folic acid ([3',5',9(n)-3H]folic acid, potassium salt, 31 Ci/mmol, TRK 212; The Radiochemical Centre, Amersham, Bucks, U.K.) and generally labelled [3H]folinic acid (specific radioactivity 2 Ci/mmol), prepared by tritium exchange labelling (The Radiochemical Centre; reference TR1) from dx-folinic acid (Lederle) and purified by the method of Nixon & Bertino (1971), were used. The purity was checked by DEAE-cellulose chromatography, by Lactobacillus casei microbiological assay and by spectral analysis. The [3H]folinic acid was kindly supplied by Dr J. Perry. Trito tiated methotrexate ([3',5',9(n)-3H]methotrexate, 9·3 Ci/mmol, TRK 224, The Radiochemical Centre) was also used as precursor. [3H]Methyltetrahydrofolate polyglutamate standard markers, prepared by labelling Lactobacillus casei folates with [3',5',9(n)-3H]folic acid (Buehring, Tamura & Stokstad, 1974), were kindly supplied by Dr E. L. R. Stokstad, Dr K. U. Buehring and Dr J. Perry. These were purified by DEAE-cellulose chromatography and de-salted with Sephadex G-25. At the end of the incubation period, the cells were harvested by centrifugation at 2000 rev./min for 5 min at 4°C, washed three times with cold (4°C) TC 199 (Wellcome) and suspended in 3 ml of potassium phosphate buffer (0·1 mol/l), pH 8·5, containing 0·057 mol of ascorbic acid/l and 0·2 mol of 2-mercaptoethanol/l and autoclaved at 115°C for 5 min. The extract was centrifuged; the supernatant, together with one wash of the protein deposit with 1 ml of potassium phosphate buffer, was stored at 4°C. Further washing of the protein deposit extracted less than 1% of the total d.p.m. A quantity (1 mg) of non-radioactive folic acid, folinic acid or methotrexate to act as a column marker was added before adding 16 ml of potassium permanganate (0·127 mol/l) at pH 9·0 to cleave the folates at the C-9-N-10 bond as described by Houlihan & Scott (1972). The resulting labelled p-aminobenzo-yglutamate derivatives were chromatographed on a DEAE-cellulose column with a potassium chloride gradient (Brown et al., 1974a). One hundred fractions were collected and the radioactivity in each was counted (Lavoie et al., 1974), and the positions of the cold marker checked by
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Fig. 1. DEAE-cellulose chromatography of labelled human phytohaemagglutinin-stimulated lymphocytes incubated for 72 h with \([{}^3H]\)folinic acid. \([{}^3H]\)Folic acid (2 \(\mu\)Ci) was added to each 3 ml culture bottle containing \(3 \times 10^6\) normal lymphocytes and phytohaemagglutinin. In this experiment, a total of \(21 \times 10^6\) lymphocytes were cultured. After 72 h incubation at 37°C, the cells were harvested; folate was extracted, cleaved by potassium permanganate and chromatographed. Vertical scale = d.p.m. from 2.5 ml of each 3.8 ml fraction. The positions of standard folate polyglutamate markers are indicated. Methyltetrahydrofolate diglutamate, after cleavage, chromatographed with a peak at tube 42. Total d.p.m. applied to the column = 45 405; total d.p.m. eluting before gradient = 6114; total d.p.m. eluting during the gradient = 37 580; total d.p.m. eluting before the Glu_1 marker = 3006.

Table 1. Composition of labelled cell folate after incubation of phytohaemagglutinin-stimulated lymphocytes with \([{}^3H]\) folic acid or \([{}^3H]\)folinic acid for 0–72 h

Results are given as \% of total recovered radioactivity chromatographing during gradient elution. Total d.p.m. applied to the DEAE column ranged from 21 222 to 70 070 for \([{}^3H]\) folic acid and from 11 384 to 64 868 for \([{}^3H]\) folinic acid.

<table>
<thead>
<tr>
<th>Glutamate chain length</th>
<th>Mean ± SD</th>
<th>Glutamate chain length</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>[{}^3H]Folic acid</td>
<td></td>
<td>[{}^3H]Folinic acid</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4–42</td>
<td>22.4 ± 15.6</td>
<td>2–30</td>
</tr>
<tr>
<td>2</td>
<td>1–3</td>
<td>2.2 ± 0.7</td>
<td>2–20</td>
</tr>
<tr>
<td>3</td>
<td>4–17</td>
<td>12.7 ± 4.4</td>
<td>9–16</td>
</tr>
<tr>
<td>4</td>
<td>7–26</td>
<td>20.5 ± 6.6</td>
<td>10–20</td>
</tr>
<tr>
<td>5</td>
<td>15–25</td>
<td>20.1 ± 4.2</td>
<td>23–40</td>
</tr>
<tr>
<td>6</td>
<td>8–22</td>
<td>15.6 ± 4.4</td>
<td>4–12</td>
</tr>
<tr>
<td>7</td>
<td>0–13</td>
<td>5.7 ± 5.2</td>
<td>1–17</td>
</tr>
<tr>
<td>8</td>
<td>0–4</td>
<td>1.1 ± 1.7</td>
<td>0–1</td>
</tr>
</tbody>
</table>

Label chromatographing before peak Glu_1 3–24 8.9 ± 7.6 7–19 15.1 ± 3.8
\% of total d.p.m. applied to column recovered during gradient elution 63–93 79.7 ± 11.1 47–77 66.6 ± 12.1

spectral analysis at 260 nm and 280 nm. The positions of the labelled standard markers after potassium permanganate oxidation were determined to separate column runs, an identical gradient made with an LKB Ultragrad gradient marker being used. Plasma contains an enzyme, folate conjugase, pH
optimum 4.6, which hydrolyses folate polyglutamates to folate monoglutamates. Further evidence for the polyglutamate nature of the peaks of radioactivity chromatographing after the monoglutamate peak was obtained by pre-incubating the labelled cell extract with fresh human plasma, pH 4.6, before permanganate cleavage and chromatography. The peaks considered to be polyglutamate derivatives were invariably removed from the subsequent chromatographic pattern.

For time-course studies, \( [\text{3H}] \) folic acid or \( [\text{3H}] \) folinic acid was added at times 0, 48 and 68 h before harvest of the cells at 72 h. In order to examine the effects of methotrexate on polyglutamate formation, methotrexate (10 \( \mu \)mol/l) was added to cultures at 45 h and either \( [\text{3H}] \) folic acid or \( [\text{3H}] \) folinic acid was added at 48 h. The control cultures and the cultures to which methotrexate had been added were then harvested at 72 h.

**Results**

**Formation of folate polyglutamate derivatives**

Fig. 1 shows a typical elution pattern of radioactivity from human lymphocytes after 72 h of incubation with \( [\text{3H}] \) folic acid, with subsequent permanganate oxidation. The major peaks occur at positions corresponding to those of markers of 1, 4, 5 or 6 glutamate chain length. Other peaks are present at positions 2, 3, 7 and 8 glutamate chain length. Table 1 summarizes the results of seven similar experiments. Approximately equal (20.1–22.4\%\) proportions of mono-, tetra- and penta-glutamate are present. There are also substantial amounts of labelled tri- and hexa-glutamate. For the purpose of expressing a proportion of the total radioactivity in each peak, the radioactivity which chromatographed before the monoglutamate peak, which amounted to 8.9 ± 7.6\% of the total radioactivity recovered from the column (Table 1), has been ignored. The exact identity of this early eluting radioactivity, presumably derived from breaking-down folates, is uncertain. The recovery of radioactivity from the columns during the gradient averaged 79.7\%; the remaining radioactivity largely left the column before commencement of the gradient.

When \( [\text{3H}] \) folinic acid was incubated with cells for 72 h, the proportion of labelled folates in the different polyglutamate peaks was different (Table 1). Folate pentaglutamates formed by far the largest peak (mean 31.8\%\) with smaller (between 10.7 and 16.0\%\) quantities of tetra-, tri-, di- and monoglutamate and only 7.3\% hexa- and septa-glutamate. The proportion of labelled folate monoglutamate in the cell was also less than after incubation of the cells for 72 h with \( [\text{3H}] \) folic acid (Tables 1 and 2).

Table 2 summarizes the data for the time-course of formation of labelled cell folates from labelled folic acid and folinic acid. Each point is the mean value from six culture bottles. With \( [\text{3H}] \) folic acid in the external medium and after 4 h, nearly 90\% of the radioactively labelled folate in the cells is still in the monoglutamate form but small amounts of polyglutamates of folate, up to the septaglutamate, are also present. After 24 h incubation, substantially less folate monoglutamate remains and increased proportions of folate polyglutamate derivatives are present. After 72 h, the pattern is similar but with the proportion of penta-, hexa- and septa-glutamate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time of incubation (h)</th>
<th>Radioactivity (d.p.m./10^6 cells)</th>
<th>% of d.p.m. added to column</th>
<th>Radioactivity eluting during the gradient (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glu1</td>
</tr>
<tr>
<td>( [\text{3H}] ) Folic acid</td>
<td>0–72</td>
<td>15833</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>48–72</td>
<td>10417</td>
<td>66</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>68–72</td>
<td>5000</td>
<td>65</td>
<td>86</td>
</tr>
<tr>
<td>( [\text{3H}] ) Folinic acid</td>
<td>0–72</td>
<td>20513</td>
<td>63</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>48–72</td>
<td>20079</td>
<td>57</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>68–72</td>
<td>8613</td>
<td>64</td>
<td>26</td>
</tr>
</tbody>
</table>
somewhat higher and the proportion of mono-, di- and tetra-glutamate somewhat lower. There was considerably more rapid formation of folate polyglutamate derivatives with [3H]folinic acid in the external medium, and after 4 h the proportions of labelled higher polyglutamates was only slightly less than after 24 h or 72 h incubation periods (Table 2).

**Formation of methotrexate polyglutamate derivatives**

Table 3 shows the results of four experiments in which labelled methotrexate has been incubated with phytohaemagglutinin-stimulated lymphocytes between 48 and 72 h of culture. Small quantities of radioactivity chromatographing in the positions of the di- (4-5%), tri- (1-5%) and tetra-glutamate (1%) markers and traces of label chromatographing as pentaglutamate derivatives of methotrexate were present. The polyglutamate nature of these derivatives was confirmed by pre-incubation of the lymphocyte extracts with plasma conjugase, which completely eliminated the peaks after the glutamate 1 (Glut) marker but did not remove the peak chromatographing as Glut1.

**Effect of methotrexate on formation of polyglutamate derivatives from labelled folic acid and folinic acids**

In these experiments, non-radioactive methotrexate (10 mmol/l) was added at 45 h of culture,
labelled folic acid or folinic acid was added at 48 h and the folates were extracted at 72 h. Some polyglutamate derivatives were formed from [3H]folic acid in the presence of methotrexate (Table 4). The total uptake of [3H]folic acid into the cells was diminished to approximately a third by methotrexate (Table 4), however, and the only radioactive peaks considered to be polyglutamate derivatives were distinct peaks of tri- and tetra-glutamate and a trace of di-glutamate. No label chromatographing as higher folate polyglutamate derivatives was detectable. On the other hand, when labelled folinic acid was used as substrate for folate polyglutamate synthesis, methotrexate had no effect on the formation of polyglutamate derivatives and the uptake of labelled folinic acid was greater in the presence of methotrexate than in control cultures (Table 4).

Discussion

The results of this study show that human phytohaemagglutinin-stimulated lymphocytes are able to form derivatives from labelled folic acid, labelled folinic acid and from labelled methotrexate, which, after heat extraction and permanganate oxidation, chromatographed on DEAE-cellulose in positions corresponding to similarly treated folate polyglutamate markers. These peaks, which are removed by prior incubation at pH 4-6 with plasma, are considered to be polyglutamate derivatives of the labelled precursors. The studies with folic acid as substrate show that after 72 h approximately equal quantities of mono-, tetra-, penta- and hexa-glutamate are present in lymphocytes. It is unlikely, however, that this pattern is identical with that of the endogenous lymphocyte folates since the labelled precursor was left in contact with the cells until the time of extraction. It is apparent from the time-course studies that cells are able to accumulate [3H]folic acid more rapidly than they are able to convert this into polyglutamate derivatives. A major part of the delay may be due to delay in reduction of folic acid to tetrahydrofolate since when identical experiments were carried out with a fully reduced folate, folinic acid (5-formyltetrahydrofolate), as precursor, much smaller quantities of labelled monogluthamate could be detected after 72 h. Moreover, the pattern of the polyglutamate derivatives that were found after incubation with [3H]folinic acid for as short a period as 4 h showed only slightly less of the incorporated labelled folate in the higher folate polyglutamate fractions compared with 24 or 72 h incubation results. Previous studies in animals have all used labelled folic acid as precursor and have suggested that it takes several days for equilibration with endogenous folate (Corrocher et al., 1972; Brown et al., 1974a, b; Leslie & Baugh, 1974). Though the present studies do not provide a study of equilibration of the labelled precursor with endogenous human folates, they do suggest that in proliferating human cells, at least, folate polyglutamate formation occurs quite rapidly, over a period of hours or possibly even over a shorter period, once the folate monoglutamate precursor has been fully reduced.

The reason for the consistent quantitative differences in the proportions of label in the folate polyglutamate peaks of different sizes with folic acid or folinic acid as precursor is difficult to explain. This does not seem to be due to an artifact produced by the permanganate oxidation technique, since using the zinc reduction technique of Baugh, Braverman & Nair (1974) to cleave the C-9-N-10 bond before chromatography gave the same differences in chromatographic patterns to those found with the oxidation technique. Part of the difference may be due to the relative speeds of incorporation of non-reduced and reduced folate into the polyglutamate fractions.

At all events, the conclusion from both labelled precursor studies that the main forms of folate in human lymphocytes and presumably in other human cells are tetra-, penta- and hexa-glutamate is consistent with the finding in other mammalian tissues (Shin et al., 1972; Houlihan & Scott, 1972; Brown et al., 1974a, b) in rat, guinea-pig, hamster and monkey, in which penta- and hexa-glutamate have been found to predominate in the liver and kidney.

The effects of methotrexate on polyglutamate formation from labelled folic acid in human lymphocytes is very similar to that reported by Corrocher & Hoffbrand (1972) in the guinea-pig. They found that some un-reduced polyglutamate derivatives of folic acid could be formed from labelled folic acid despite the presence of methotrexate. Their work has been supported by the findings of Shin et al. (1974) and Garth & Baugh (1974), though in the rat, Brown, Davidson, Weir & Scott (1974c) could not detect labelled folate polyglutamates of folic acid when animals were pretreated with methotrexate. Presumably methotrexate impairs synthesis of folate polyglutamates from folic acid because a reduced rather than oxidized folate is the preferred substrate for the enzyme folate polyglutamate synthetase that adds on...
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glutamate moieties. The alternative possibility, that methotrexate decreases synthesis of polyglutamate derivatives of folic acid by itself acting as substrate for the enzyme and thus causing competitive inhibition is less likely as methotrexate had no effect on formation of polyglutamate derivatives from folic acid. Interestingly, although methotrexate reduced the overall uptake of labelled folic acid into cells, it actually increased the uptake of labelled folinic acid.

The amount of polyglutamate derivatives of methotrexate formed in human lymphocytes over a 24 h period is relatively small. However, it may be that with long-continued administration of methotrexate in vivo substantial amounts of polyglutamate derivatives may accumulate, particularly in the slowly turning over cells of the liver and account, in part, for the long retention of methotrexate inside cells. Baugh, Krumdieck & Nair (1973) detected polyglutamate derivatives of methotrexate in the livers of rats and the erythrocytes of patients treated with methotrexate and Whitehead (1973) detected them in mouse liver. More recently, Whitehead, Perrault & Stekner (1975) have shown that, at doses of methotrexate comparable with those used in man, substantial amounts of polyglutamate derivatives are formed in both rat liver and kidney. Coward et al. (1974) have shown that dihydrofolinic reductase is more avid for the polyglutamate derivatives of dihydrofolinic acid than for dihydrofolinic acid itself and by analogy it is likely that the polyglutamate derivatives of methotrexate may be stronger antagonists of dihydrofolinic reductase than methotrexate itself.

Studies in vitro reported in abstract form only with extracts of Neurospora crassa (Sakami et al., 1973) and rat liver (Spronk, 1973) have suggested that tetrahydrofolicate rather than methyltetrahydrofolicate or any other reduced derivative is the optimum substrate for the polyglutamate-synthesizing enzyme, though in sheep liver Gawthorne & Smith (1974) did find that methyltetrahydrofolate itself could act as substrate. Studies in vitro are now necessary to determine the optimum substrate for folate polyglutamate synthesis in human cells.

Acknowledgment

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