THE EFFECT OF VITAMIN B₁₂ DEFICIENCY ON METHYLFOLATE METABOLISM AND PTEROYLPOLYGLUTAMATE SYNTHESIS IN HUMAN CELLS

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SUMMARY

1. The uptake of $^{14}$C from [methyl-$^{14}$C]methyltetrahydrofolate was significantly reduced in the phytohaemagglutinin (PHA)-stimulated lymphocytes from nine patients with untreated pernicious anaemia compared with the uptake in seven normal subjects.

2. The uptake of $^{14}$C from [$^{14}$C]methyltetrahydrofolate by the lymphocytes from seven of the patients with pernicious anaemia was consistently increased by addition of vitamin B₁₂ in vitro.

3. The proportion of $^{14}$C taken up from [$^{14}$C]methyltetrahydrofolate transferred to non-folate compounds was found to be significantly reduced in the PHA-stimulated lymphocytes from nine patients with untreated pernicious anaemia compared with the proportion transferred in the PHA-stimulated lymphocytes from seven normal subjects. Addition of vitamin B₁₂ in vitro consistently increased the transfer in vitamin B₁₂-deficient cells but had no consistent effect in normal cells.

4. Normal and vitamin B₁₂-deficient PHA-stimulated lymphocytes took up [$^{3}$H]folic acid and after 72 h incubation converted this largely into pteroylpolyglutamate forms.

5. The proportion of labelled lymphocyte folate as pteroylpolyglutamate after incubation with [$^{3}$H]folic acid was the same in vitamin B₁₂-deficient as in normal lymphocytes and the proportion of pteroylpolyglutamates formed in vitamin B₁₂-deficient lymphocytes was unaffected by addition of vitamin B₁₂ in vitro.

6. No radioactivity could be detected in pteroylpolyglutamates after incubating normal PHA-stimulated lymphocytes with [$^{14}$C]methyltetrahydrofolate for 72 h, suggesting that pteroylpolyglutamate forms of folate cannot be made directly from methyltetrahydrofolate.

7. These results are consistent with the 'methyltetrahydrofolate trap' hypothesis.
in vitamin B\textsubscript{12} deficiency. It is suggested that reduced synthesis of pteroylpolyglutamates reported by others in vitamin B\textsubscript{12}-deficient cells may be secondary to the failure of removal of the methyl group from methyltetrahydrofolate rather than to a direct effect of vitamin B\textsubscript{12} deficiency on the enzyme responsible for pteroylpolyglutamate synthesis.

8. Reduced entry of methyltetrahydrofolate into vitamin B\textsubscript{12}-deficient cells may be secondary to failure of conversion of this compound into tetrahydrofolate.

Key words: methyltetrahydrofolate, folic acid, pteroylpolyglutamates, vitamin B\textsubscript{12}, phytohaemagglutinin-transformed lymphocytes.

A variety of disturbances of folate metabolism have been described in vitamin B\textsubscript{12} deficiency. In humans, these include a high serum folate concentration (Waters & Mollin, 1961, 1963; Herbert & Zalusky, 1962), increased clearance of folic acid (PteGlu) but decreased clearance of 5-methyltetrahydrofolate (methyl-H\textsubscript{4}PteGlu, methyltetrahydrofolate) from plasma (Chanarin, Mollin & Anderson, 1958; Herbert & Zalusky, 1962; Nixon & Bertino, 1972), low erythrocyte folate (Hansen & Weinfeld, 1962; Cooper & Lowenstein, 1964; Hoffbrand, Newcombe & Mollin, 1966), low leucocyte folate (Hoffbrand & Newcomb, 1967), decreased transport of methyltetrahydrofolate into cells (Das & Hoffbrand, 1970a; Tisman & Herbert, 1973), and an increased ratio of free to total pteroylpolyglutamate content in mature erythrocytes, implying decreased synthesis of pteroylpolyglutamate forms of folate (Jeejeebhoy, Pathare & Noronha, 1965; Chanarin, Perry & Lumb, 1974).

Decreased liver folate, decreased synthesis of pteroylpolyglutamate from folic acid and an excess of methylated compared to formylated folates has also been described in the livers of vitamin B\textsubscript{12}-deficient experimental animals (Thenen & Stokstad, 1973; Smith & Osborne-White, 1973).

The only biochemical reaction known to require both vitamin B\textsubscript{12} and folate in mammalian cells is the methylation of homocysteine to methionine (Stokstad & Koch, 1967; Huennekens, 1968; Weissbach & Taylor, 1968), and it has been suggested that the defects of folate metabolism in vitamin B\textsubscript{12} deficiency are consequent on folate becoming 'trapped' as methyltetrahydrofolate through failure of this reaction (Noronha & Silverman, 1962; Herbert & Zalusky, 1962). In the present paper the results of studies of the transfer of the methyl group from labelled methyltetrahydrofolate to non-folate compounds and of pteroylpolyglutamate synthesis from methyltetrahydrofolate and from folic acid are reported. These studies provide direct evidence in favour of the methyltetrahydrofolate trap hypothesis in vitamin B\textsubscript{12} deficiency and may help to unify the observations of a methyltetrahydrofolate trap, reduced methyltetrahydrofolate transport into cells and reduced intracellular pteroylpolyglutamate formation into a single theory to explain the abnormalities of folate metabolism seen in patients with megaloblastic anaemia due to vitamin B\textsubscript{12} deficiency.

**MATERIALS AND METHODS**

_Incorporation of radioactivity from [methyl-\textsuperscript{14}C]methyltetrahydrofolate (\textsuperscript{14}C)methyl-H\textsubscript{4}PteGlu) into non-folate compounds_

Lymphocyte cultures were set up from heparinized venous blood as previously described
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(Das & Hoffbrand, 1970a), except that Trisosil-Ficoll was used to separate lymphocytes and granulocytes (Boyum, 1968). Phytohaemagglutinin (PHA) (Wellcome) was added at time zero and the cultures were incubated for 72 h. [¹⁴C]Methyl-H₄PteGlu (The Radiochemical Centre, Amersham, Bucks.; specific radioactivity 61 mCi/(mmol), a 50:50 mixture of diastereoisomers epimeric about C-6, 0.5 μCi, was added to each of the 3 ml cultures. [¹⁴C]Methyl-H₄PteGlu was dissolved in 0.2 mol/l 2-mercaptoethanol and stored in aliquots at -70°C until the day of use. The purity was checked by chromatography on DEAE-cellulose of an aliquot of the batch and of the aliquot stored for the longest time; both showed over 95% purity (Corrocher, Bhuyan & Hoffbrand, 1972). In some experiments, 7.4 nmol (10 μg) of vitamin B₁₂ (cyanocobalamin) was also added in vitro at the start of the incubation period to half of the cultures. In one experiment, bone-marrow cells from a patient with untreated pernicious anaemia were incubated with [¹⁴C]methyl-H₄PteGlu. The bone marrow culture was set up as described by Metz, Kelly, Chappinswett, Waxman & Herbert (1968), 0.5 μCi of [¹⁴C]methyl-H₄PteGlu being added to 3 x 10⁶ bone-marrow cells in a total volume of 1 ml. The cells were incubated at 37°C without shaking (lymphocytes for 72 h, bone marrow for 24 h). It is likely that some decomposition of [¹⁴C]methyl-H₄PteGlu, and to a less extent of [³H]folic acid (see below), occurred during the 72 h incubation periods, which were used to achieve significant radioactivity counts in the cells. However, the decomposition products of these compounds do not chromatograph as folate or protein in the experiments listed below. Moreover, control experiments with [¹⁴C]methyl-H₄PteGlu and [³H]folic acid deliberately allowed to decompose over weeks on the bench have both shown less than 10% uptake of radioactivity by normal PHA-stimulated lymphocytes over a 72 h period compared to experiments with chromatographically pure (>95%) materials. The cells were harvested by centrifugation three times in cold (4°C) TC 199 medium (Wellcome), suspended in cold 0.1 mol/l phosphate–citrate buffer, pH 4.6, containing 0.057 mol/l ascorbic acid and 0.2 mol/l 2-mercaptoethanol and sonicated. Polyglutamate forms of folate were deconjugated by incubation with 0.1 ml of fresh normal human plasma for 90 min since this completely converts the compounds into monoglutamate forms and the non-protein compounds were extracted by heat at 115°C for 5 min. The protein precipitate was washed twice in the phosphate–citrate buffer and the precipitate digested with 2 ml of Hyamine hydroxide (1 mol/l in methanol, Koch–Light Laboratories). The proportion of [¹⁴C] in folate and non-folate compounds in the combined washings and supernatant was measured as described by Nixon, Glutsky, Nahas & Bertino (1973) as follows: 1 ml was kept for radioactivity counting, the rest was poured through an ion-exchange column (AG-1, X8, Biorad; 2.5 cm x 0.5 cm). The eluate was collected and combined with a 3 ml wash of a 1 mmol/l solution of methionine. Folates were then eluted from the resin with 50 ml of 1 mol/l hydrochloric acid. The total radioactivity in each of the two eluates was then measured. Recovery of d.p.m. added to the AG-1 (X8) column ranged from 87 to 115% (mean 97%).

Incorporation of [³H]-labelled folic acid into pteroylpolyglutamates

In these experiments, PHA-stimulated lymphocytes were incubated for 72 h with tritiated folic acid [³',⁵',⁹-³H]folic acid (31 Ci/mmol; The Radiochemical Centre), 2 Ci (64.5 pmol)/3 ml culture. The [³H]folic acid was made up in distilled water, stored in aliquots at -70°C and
the purity checked by DEAE-cellulose column chromatography (Corrocher et al., 1972) on the initial sample and final aliquot (both of which showed over 95% purity). The cells were then washed three times with cold (4°C) TC 199 medium and resuspended in 0-1 mol/l potassium phosphate buffer, pH 8.5, containing 1 g of ascorbic acid/100 ml and 0-2 mol/l 2-mercaptoethanol, and heat-extracted at 115°C for 5 min. The labelled folates in the supernatant were treated with 4 ml of potassium permanganate solution (126 mmol/l) to digest folates at the C-9–N-10 bond before DEAE-cellulose column chromatography of the p-aminobenzoylglutamate fragments, according to the number of glutamate moieties remaining as described by Houlihan & Scott (1972).

<table>
<thead>
<tr>
<th>TABLE 1. Haematological data for the ten patients with untreated pernicious anaemia and seven control subjects</th>
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<tr>
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<td>---------------------------------------------------------------</td>
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<tr>
<td><strong>Pernicious anaemia</strong> (lymphocytes)</td>
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<td>1</td>
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<td>9</td>
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<tr>
<td><strong>Bone marrow</strong></td>
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<tr>
<td>10</td>
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<tr>
<td><strong>Normal subject</strong> (lymphocytes)</td>
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</table>

**Incorporation of radioactivity from [¹⁴C]methyl-H₄PteGlu into pteroylpolyglutamates**

Experiments were performed as described in the preceding paragraph, except that the cells were incubated for 72 h with 0.5 µCi of [¹⁴C]methyl-H₄PteGlu/3 ml culture. After washing and heat-extraction the extract was poured through a DEAE-cellulose column and folates were eluted with a phosphate buffer gradient as described by Corrocher et al. (1972). The radioactivity in the eluates was measured.
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Liquid-scintillation counting

The counting liquid consisted of Triton X100 500 ml, toluene 1 ml, POPOP (1,4-bis-(5-phenyloxazol-2-yl)benzene) 0.45 g, PPO (2,5-diphenyloxazole) 6 g, to 10 ml of which 1 ml of the sample was added. Counts were converted from c.p.m. into d.p.m. by use of an internal standard ([¹⁴C]toluene, 0.502 μCi of [¹⁴C]g; The Radiochemical Centre).

Subjects

Studies were made on the lymphocytes of nine patients with untreated pernicious anaemia. The marrow from a tenth patient was also studied (Table 1). The patients comprised four males and six females. Their haemoglobin concentrations ranged from 5.1 to 10.5 g/dl and all showed florid megaloblastic marrow appearances with adequate iron stores, and malabsorption of vitamin B₁₂ corrected by intrinsic factor. The serum vitamin B₁₂ concentrations ranged from 5.9 to 70.8 pmol/l (8-96 ng/l) and serum folate concentrations from 8.4 to 48 nmol/l (3.7-21.2 μg/l). Cells were also obtained from seven normal healthy adult volunteers, all with normal serum vitamin B₁₂ and folate and haemoglobin concentrations (Table 1).

RESULTS

Transfer of [¹⁴C] from [¹⁴C]methyl-H₄PteGlu to non-folate compounds

There was significantly lower uptake of [¹⁴C] from [¹⁴C]methyl-H₄PteGlu by the vitamin B₁₂-deficient lymphocytes compared with normal lymphocytes (P<0.005; t-test) (Table 2). Addition of vitamin B₁₂ in vitro significantly increased the uptake in the vitamin B₁₂-deficient cells (P<0.005; paired t-test) but had no consistent effect in the four normal cell samples tested. The proportions of [¹⁴C] recovered as folate, protein and non-folate, non-protein compounds in the PHA-stimulated lymphocytes and bone marrow are also shown in Table 2. The proportion recovered as folate in the nine patients with pernicious anaemia ranged from 28.8 to 56.0% (mean 39.4%). This was significantly greater than the proportion of [¹⁴C] recovered as folate in the lymphocytes from the seven normal subjects (range 9.1-26.0, mean 15.0%) (P<0.001). In the seven pernicious anaemia patients tested, addition of vitamin B₁₂ in vitro significantly reduced both the amount and proportion of [¹⁴C] remaining as folate (t-test for paired samples, P<0.01 and <0.05 respectively) and significantly increased the amount recovered as protein (t-test for paired samples, P<0.001). Vitamin B₁₂ added in vitro had no consistent effect on the proportion of [¹⁴C] recovered as non-folate, non-protein compounds in the vitamin B₁₂-deficient cells or on the proportion recovered as folate, protein, and non-folate, non-protein compounds in normal cells (Table 2).

Synthesis of pteroylpolyglutamate from [³H]folic acid: effect of vitamin B₁₂ deficiency

This was studied in the PHA-stimulated lymphocytes from four normal and four vitamin B₁₂-deficient patients. The proportion of the incorporated radioactivity as pteroylpolyglutamates in the four normal PHA-stimulated lymphocytes ranged from 85 to 92% (mean 88%). After KMnO₄ digestion, the p-aminobenzoylglutamate fragments chromatographed
TABLE 2. Total uptake and distribution of \(^{14}C\) in PHA-stimulated lymphocytes and bone marrow after incubation of cells with \(^{14}C\)methyltetrahydrofolate

<table>
<thead>
<tr>
<th></th>
<th>Total recovered (d.p.m.)</th>
<th>Proportion of total recovered radioactivity</th>
<th>Non-protein, non-folate [% (d.p.m.)]</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(d.p.m.)</td>
<td>Folate [% (d.p.m.)]</td>
<td>Protein [% (d.p.m.)]</td>
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<tr>
<td></td>
<td>Alone + B(_{12})</td>
<td>Alone + B(_{12})</td>
<td>Alone + B(_{12})</td>
</tr>
<tr>
<td>Pernicious anaemia (lymphocytes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3697 4579</td>
<td>56.0 (2070) 28.3 (1295)</td>
<td>27.5 (1016) 50.4 (2308)</td>
</tr>
<tr>
<td>2</td>
<td>2750 5126</td>
<td>52.7 (1450) 26.6 (1363)</td>
<td>34.2 (940) 60.0 (3071)</td>
</tr>
<tr>
<td>3</td>
<td>2836 4965</td>
<td>42.4 (1210) 21.8 (1074)</td>
<td>34.6 (988) 48.3 (2397)</td>
</tr>
<tr>
<td>4</td>
<td>3484 4139</td>
<td>41.6 (1450) 11.7 (485)</td>
<td>37.7 (1309) 77.1 (3192)</td>
</tr>
<tr>
<td>5</td>
<td>1599 —</td>
<td>39.4 (630) —</td>
<td>48.8 (780) —</td>
</tr>
<tr>
<td>6</td>
<td>1102 —</td>
<td>32.4 (357) —</td>
<td>57.7 (636) —</td>
</tr>
<tr>
<td>7</td>
<td>7968 8688</td>
<td>31.4 (2500) 22.4 (1944)</td>
<td>43.4 (3258) 52.5 (4560)</td>
</tr>
<tr>
<td>8</td>
<td>3925 4415</td>
<td>30.6 (1200) 18.1 (800)</td>
<td>54.9 (2155) 68.3 (3015)</td>
</tr>
<tr>
<td>9</td>
<td>2184 4588</td>
<td>28.8 (629) 11.6 (563)</td>
<td>42.5 (928) 66.3 (3221)</td>
</tr>
<tr>
<td>Mean</td>
<td>3285 5253</td>
<td>39.5 (1277) 20.0 (1074)</td>
<td>42.4 (1367) 60.4 (3109)</td>
</tr>
<tr>
<td>SD</td>
<td>1995 1552</td>
<td>9.8 (696) 6.6 (512)</td>
<td>10.0 (929) 10.7 (740)</td>
</tr>
<tr>
<td>SEM</td>
<td>665 587</td>
<td>3.3 (232) 2.5 (193)</td>
<td>3.3 (310) 4.0 (280)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>10 21820 28566</td>
<td>62.5 (13645) 46.3 (13235)</td>
<td>19.2 (4180) 33.8 (9668)</td>
</tr>
<tr>
<td>Normal (lymphocytes)</td>
<td></td>
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<td></td>
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<tr>
<td>1</td>
<td>8641 —</td>
<td>26.0 (2250) —</td>
<td>40.5 (3400) —</td>
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<td>2</td>
<td>11074 —</td>
<td>17.6 (1950) —</td>
<td>55.2 (6118) —</td>
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<tr>
<td>3</td>
<td>3558 3363</td>
<td>17.1 (610) 24.2 (815)</td>
<td>60.8 (2163) 53.9 (1813)</td>
</tr>
<tr>
<td>4</td>
<td>7272 7767</td>
<td>14.1 (1025) 15.2 (1182)</td>
<td>66.6 (4844) 64.5 (5016)</td>
</tr>
<tr>
<td>5</td>
<td>6878 6891</td>
<td>11.2 (770) 5.1 (351)</td>
<td>79.0 (5436) 87.8 (6051)</td>
</tr>
<tr>
<td>6</td>
<td>5183 4139</td>
<td>10.0 (520) 11.7 (485)</td>
<td>80.0 (4144) 77.1 (3192)</td>
</tr>
<tr>
<td>7</td>
<td>9620 —</td>
<td>9.1 (880) —</td>
<td>83.1 (7994) —</td>
</tr>
<tr>
<td>Mean</td>
<td>7461 5542</td>
<td>15.0 (1144) 14.0 (708)</td>
<td>66.4 (4886) 70.8 (4018)</td>
</tr>
<tr>
<td>SD</td>
<td>2579 2123</td>
<td>5.9 (680) 8.0 (371)</td>
<td>15.5 (1887) 14.8 (1886)</td>
</tr>
<tr>
<td>SEM</td>
<td>975 1062</td>
<td>2.2 (257) 4.0 (186)</td>
<td>5.9 (773) 7.4 (943)</td>
</tr>
</tbody>
</table>
just in front of KMnO₄-treated synthetic pteroylhexaglutamic acid (Fig. 1). Labelled pteroylpolyglutamates chromatographing in a position identical with labelled compounds synthesized in normal cells were synthesized from [³H]folic acid in vitamin B₁₂-deficient lymphocytes from all four patients, and the proportion of pteroylglutamate among the labelled cell folate was similar (range 84-93%, mean 87%) to normal and was not significantly altered by addition of vitamin B₁₂ in vitro at the beginning of the 72 h cultures in any of the four cases (Fig. 1).

![Graph of DEAE-cellulose column chromatography](image)

**Fig. 1.** DEAE-cellulose column chromatography of untreated pernicious anaemia lymphocytes labelled with [³H]folic acid after treatment with 2% potassium permanganate (see the text). Typical results from one of four patients. (a) Results with vitamin B₁₂ added at the start of culture; (b) results without added vitamin B₁₂. ○, Radioactivity of hydrolysed labelled lymphocyte folate; ○, extinction of hydrolysed synthetic pteroylhexaglutamic acid (PteGlu₆) standard [prepared by Dr J. Perry, Northwick Park Hospital, by the method of Krumdieck & Baugh (1969)].

**Synthesis of pteroylpolyglutamates from [¹⁴C]methyl-H₄PteGlu**

In none of three experiments with normal lymphocytes and one experiment with vitamin B₁₂-deficient lymphocytes from case 10 could ¹⁴C-labelled pteroylpolyglutamates be detected after incubation of the PHA-stimulated lymphocytes with [¹⁴C]methyl-H₄PteGlu for 72 h. The radioactivity eluted entirely as a single peak corresponding to standard [¹⁴C]methyl-H₄PteGlu (Fig. 2).
FIG. 2. DEAE-cellulose column chromatography of normal lymphocytes labelled with [\(^{14}\)C]-methyltetrahydrofolate. A single peak at tube 17 corresponding to standard \([^{14}\)C]methyltetrahydrofolate (\([^{14}\)C]methyl-\(\text{H}_4\text{PteGlu}\)) was observed and no radioactivity was detected in the pteroyldi-, pteroyltri- or pteroylpolyglutamate regions (tubes 26-75; Corrocher et al., 1972).

DISCUSSION

The results of these studies confirm previous reports that uptake of methyltetrahydrofolate by vitamin \(B_{12}\)-deficient cells is subnormal and can be corrected by addition of vitamin \(B_{12}\) in vitro (Das & Hoffbrand, 1970a; Tisman & Herbert, 1973). They also give direct support for the concept that vitamin \(B_{12}\) deficiency causes a trapping of folate in the methyltetrahydrofolate form in human cells, as also shown by Chello & Bertino (1973) for L5178Y cells, and suggest how such a trap might explain reduced formation of other intracellular folates, including pteroylpolyglutamate forms, in vitamin \(B_{12}\) deficiency.

The studies of transfer of the methyl group from methyltetrahydrofolate to non-folate compounds show that this transfer is consistently decreased in the cells of vitamin \(B_{12}\)-deficient patients compared with normal cells, and can be partly or completely corrected in vitamin \(B_{12}\) deficiency by addition of vitamin \(B_{12}\) in vitro. The transfer of the methyl group to protein was also consistently increased by addition of vitamin \(B_{12}\) in vitro, presumably owing to increased formation of methionine. The differences, though consistent and significant, were not great and might seem insufficient to explain a major disturbance of intracellular folate metabolism. However, the synthesis and activity of the enzyme 5,10-methylenetetrahydrofolate reductase, which synthesizes 5-methyltetrahydrofolate, and of the enzyme 5-methyltetrahydrofolate–homocysteine methyltransferase may be modified by a number of intracellular metabolites including homocysteine, methionine and \(S\)-adenosylmethionine (Kutzbach & Stokstad, 1967; Kamely, Littlefield & Erbe, 1973), and the effect of a block due to vitamin \(B_{12}\) deficiency in conversion of homocysteine into methionine on the levels of these intermediates in human marrow and other cells may be important in determining how much tetrahydrofolate can be formed from methyltetrahydrofolate inside these cells. For instance, Thenen & Stokstad (1973) and Smith, Osborne-White & Gawthorne (1974) observed that methionine added to the diet substantially increased pteroylpolyglutamate synthesis in vitamin \(B_{12}\)-deficient rats and sheep respectively. Thenen & Stokstad (1973) attributed their results to conversion of methionine into \(S\)-adenosylmethionine, which then inhibited methylenetetra-
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hydrofolate reductase (Kutzbach & Stokstad, 1967) and so maintained folate in the tetra-
hydrofolate and 10-formyltetrahydrofolate states. Noronha & Silverman (1962) originally
showed that added dietary methionine does indeed reduce the proportion of 5-methylfolate
derivatives in vitamin B₁₂-deficient rat liver and increases 10-formylfolate derivatives.

An alternative theoretical explanation for the results here could be proposed, based on the
assumptions: (a) that human lymphocyte and bone-marrow cells take up significant amounts
of the inactive as well as the biologically active diastereoisomer of methyltetrahydrofolate,
(b) that lack of vitamin B₁₂ only reduces uptake of the active diastereoisomer, and (c) that
only the active isomer is capable of transferring its [¹⁴C]methyl group to protein. Weir,
Brown, Freedman & Scott (1973) have shown that passive transfer of the inactive form of
[¹⁴C]methyltetrahydrofolate may occur across the human small intestinal mucosa. The relative
uptake of active and inactive isomers of methyltetrahydrofolate by human haemopoietic cells
has not been studied and this theory therefore cannot be completely ruled out. It is, however,
a less attractive explanation for the present results on ¹⁴C transfer from [¹⁴C]methyltetra-
hydrofolate, since in each case of pernicious anaemia studied, the absolute radioactivity
counts recovered as folate fell on addition of vitamin B₁₂.

Folate enters human marrow and other cells in vivo in the reduced monoglutamate form,
methyltetrahydrofolate, which has been produced from ingested natural folates by the in-
testinal mucosal cells (Perry & Chanarin, 1970). Over 90% of intracellular folate is, however,
in the pteroylpolyglutamate form, methylated, formylated and with other single carbon atom
substitutions. Cells must therefore be capable of converting monoglutamate forms of folate
into polyglutamate forms. The results here suggest, however, that the methyltetrahydrofolate
which enters the cell is not a direct substrate for pteroylpolyglutamate formation in human
cells, since no [¹⁴C]methylated pteroylpolyglutamates could be detected from [methyl-¹⁴C]-
methyltetrahydrofolate. The alternative explanation for the observation in Fig. 2, that methyl-
atated pteroylpolyglutamates are formed directly from methyltetrahydrofolate and the [¹⁴C]-
methyl groups are then rapidly and completely removed, seems unlikely in view of the high
proportion of pteroylpolyglutamates which are methylated in mammalian cells (Corrocher
et al., 1972; Thenen & Stokstad, 1973). It is more likely that a monoglutamate form of folate
other than methyltetrahydrofolate is the natural substrate for pteroylpolyglutamate for-
mation in human cells. Griffin & Brown (1964) showed that in Escherichia coli di- or tetra-
hydrofolate is the preferred substrate for this reaction and it is probable that highly reactive
tetrahydrofolate is the normal substrate in human cells for pteroylpolyglutamate formation.
Chanarin et al. (1974) have postulated that vitamin B₁₂ is needed directly for pteroylpoly-
glutamate synthesis in human cells. The alternative hypothesis is suggested here that pteroyl-
polyglutamate formation may be less than normal in vitamin B₁₂ deficiency, not because of
direct involvement of vitamin B₁₂ in pteroylpolyglutamate synthesis but rather because the
'methyltetrahydrofolate trap' leads to reduced supply of tetrahydrofolate, the correct sub-
strate for pteroylpolyglutamate formation (Fig. 3). The ability of vitamin B₁₂-deficient cells
to synthesize pteroylpolyglutamates normally from folic acid shown here fits with the view
that there is no intrinsic defect of polyglutamate formation in vitamin B₁₂-deficient cells.

The twin observations of relative failure of vitamin B₁₂-deficient cells to take up methyl-
tetrahydrofolate with normal uptake of folic acid in vitro (Das & Hoffbrand, 1970a; Tisman &
Herbert, 1973) and slow clearance in vivo of methyltetrahydrofolate but rapid clearance of
folic acid (Chanarin et al., 1958; Herbert & Zalusky, 1962; Nixon & Bertino, 1972) are con-
sistent with the hypothesis that incorporation of folic acid into pteroylpolyglutamates is normal in human vitamin B\textsubscript{12}-deficient cells, whereas pteroylpolyglutamate formation from methyltetrahydrofolate is defective (Fig. 3). The normal monoglutamate pool of folate found in the cells of vitamin B\textsubscript{12}-deficient rats (Thenen & Stokstad, 1973) and humans (Chanarin et al., 1974) rather than an increased pool as might be expected on the 'methyltetrahydrofolate trap' hypothesis may be due to inability of cells to retain excess amounts of the principal monoglutamate form, 5-methyltetrahydrofolate. Conversion of folates from monoglutamate into polyglutamate forms is probably necessary for retention in the cells (Brown, Davidson & Scott, 1974) and excess excretion of 5-methyltetrahydrofolate from cells in vitamin B\textsubscript{12} deficiency could maintain the intracellular pool of monoglutamate constant.

![Diagram of folate metabolism](image)

Fig. 3. Proposed site of block in pteroylpolyglutamate formation from methyltetrahydrofolate (methyl-H\textsubscript{4}PteGlu) in vitamin B\textsubscript{12} deficiency (*). PteGlu = folic acid; H\textsubscript{2}PteGlu = dihydrofolate; H\textsubscript{4}PteGlu = tetrahydrofolate; H\textsubscript{4}PteGlu\textsubscript{2} or 3, etc. = tetrahydropteroylidi- or triglutamate, etc.

For vitamin B\textsubscript{12}-dependent methionine synthesis methyltetrahydrofolate, a monoglutamate, rather than the polyglutamate derivative, is likely to be the active coenzyme in mammalian systems (Guest, Friedman, Dilworth & Woods, 1964; Mangum, Murray & North, 1969). For other folate-requiring reactions, pteroylpolyglutamates may be the natural coenzymes and monoglutamates only useful models of these reactions (Blakley, 1957, 1959; Rabino-witz, 1960). The results of Shin, Williams & Stokstad (1972) and Houlihan & Scott (1972) have shown that pteroylpentaglutamates are the main forms in rat liver and our results confirm that pteroylpenta- and pteroylhexa-glutamates predominate in human lymphocytes.
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(Mavoie & Hoffbrand, 1974, and unpublished observations). The predominance of these forms of folate in rapidly proliferating primitive cells as well as in resting cells in both humans and rats suggests that these pteroylpolyglutamates may form the natural folate coenzymes needed for reaction in purine and thymidine synthesis (Lavoie, Tripp, Parsa & Hoffbrand, 1975).

Thymidylate synthesis is defective in vitamin B₁₂-deficient marrow and lymphocytes (Metz et al., 1968; Das & Hoffbrand, 1970b) and this can be corrected by vitamin B₁₂ and folic acid, but not by methyltetrahydrofolate, and the defect can be made worse by methionine and reduced by homocysteine (Metz et al., 1968; Das & Hoffbrand, 1970b; Waxman, Metz & Herbert, 1969). These observations are consistent with the present hypothesis that vitamin B₁₂ deficiency may cause reduced DNA synthesis by the sequence of reduced synthesis of the probable natural coenzyme for thymidylate synthetase, 5,10-methylenetetrahydropteroyl-penta- (or hexa-)glutamate, due to a lack of tetrahydrofolate for polyglutamate formation due to trapping of folate in the monoglutamate form of methyltetrahydrofolate (Fig. 3). Failure of serine-glycine interconversion (DeGrazia, Fish, Pollycove & Wallerstein, 1972; Ellegaard & Esmann, 1973), deranged purine metabolism (Herbert, Streiff, Sullivan & McGeer, 1964) and excessive excretion of formiminoglutamic acid (Spray & Witts, 1959; Chanarin, 1960; Kohn, Mollin & Rosenbach, 1961; Zalusky & Herbert, 1961) could all be due to lack of formation of the relevant pteroylpolyglutamate coenzymes, as also proposed by Chanarin et al. (1974). Reduced interconversion of pteroylmonoglutamate forms due to the 'methyltetrahydrofolate trap' could also deprive the cells of monoglutamate coenzyme forms where these are the natural coenzymes in human cells.

The failure of patients with vitamin B₁₂ deficiency to respond to physiological doses of folic acid (Marshall & Jandl, 1960) suggests defective utilization of folic acid itself in vitamin B₁₂ deficiency. A number of explanations for this are possible, despite the normal pteroylpolyglutamate formation from folic acid in vitamin B₁₂-deficient cells shown here. It may be that conversion in vivo of the small doses of folic acid into methyltetrahydrofolate occurs in the intestine or liver with the subsequent inability of the vitamin B₁₂-deficient bone marrow to utilize methyltetrahydrofolate. Displacement of methyltetrahydrofolate from the liver by folic acid may also prevent the marrow cells from obtaining folic acid unchanged (Chanarin & McLean, 1967). Larger doses of folic acid cross the intestine and circulate partly unchanged, and these might be expected to enter the bone marrow as such and to produce a haematological response after conversion inside cells into folate coenzymes. It may also be that vitamin B₁₂ deficiency causes a disturbance in interconversion of folate coenzymes at the pteroylpolyglutamate level, as suggested by the observation of Thenen & Stokstad (1973) in the rat, or has some other as yet unrecognized effect on pteroylpolyglutamate metabolism. Further measurements are now needed of the intracellular concentrations and turnover of specific folate monoglutamate and polyglutamate coenzymes in vitamin B₁₂ deficiency to investigate these possibilities.

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