**B₁₂ Dependence of Cell Uptake of Serum Folate: An Explanation for High Serum Folate and Cell Folate Depletion in B₁₂ Deficiency**

By Glenn Tisman and Victor Herbert

Bone marrow cells from patients with vitamin B₁₂ deficiency took up much less N⁵-methyltetrahydrofolate (MeTHF) than did normal bone marrow cells but showed no significant reduction in pteroylglutamate (PGA) uptake. Addition of B₁₂ to the incubation medium enhanced the cell uptake of MeTHF only in B₁₂-deficient patients. These data support the concept of a B₁₂ requirement for cell uptake of MeTHF.

Because of prior evidence, the current experiments were designed to test the hypothesis that vitamin B₁₂ may be required for cellular uptake of N⁵-methyltetrahydrofolate (MeTHF), which is the dominant form of folate in human serum.

**MATERIALS AND METHODS**

The methodology is essentially that of Corcino et al., 10–20 ml of bone marrow from each normal volunteer or patient with deficiency of vitamin B₁₂ or of folic acid were aspirated into 10 ml of 0.06 M Tris-buffered Hanks' balanced salt solution (THBSS), pH 7.4, containing 200 U/ml each of penicillin and streptomycin to which had been added 100 mg of heparin. The bone marrow was washed free of serum and treated as previously described for short-term (4-hr) culture. Nucleated bone marrow cells, 2–12 X 10⁶, were distributed into tubes, and the final volume was adjusted to 0.8 ml with THBSS. One-tenth milliliter of THBSS was added to each sample; in appropriate samples this THBSS contained either 5 µg nonradioactive pteroylglutamic acid (PGA) (Lederle Labs., Pearl River, N. Y.), 5 µg MeTHF (Sigma, St. Louis, Mo.), 17.5 ng methotrexate (MTX) (Lederle Labs.), or 1 µg cyanocobalamin (B₁₂) (Elkins-Sinn, Cherry Hill, N. J.). After 1 hr of preincubation at 37°C, all volumes were made up to 0.9 ml with additional THBSS. High specific activity, tritium-labeled MeTHF (prepared by Dr. Peter Nixon in Dr. Joseph Bertino's laboratory) (specific activity 11.5 Ci/mM) or tritium-labeled PGA (specific activity 34.5 Ci/mM) was then added as 17.5 ng of folate in 0.1 ml of 0.9% NaCl followed by 3 hr of incubation at 37°C. Viability of the system was determined by continued ability of the cells to incorporate ³HTdR into DNA.

Incubation was terminated by washing with Hanks' Balanced Salt Solution (HBSS).
The procedure differed slightly from that of Corcino et al.\(^3\) in that absorbed radioactive folate was extracted from the thrice-washed bone marrow cells into 3 ml rather than 1 ml of distilled water by autoclaving for 15 min at 15 lb of pressure. After autoclaving, 0.2 ml of the centrifuged (500 g for 20 min) supernate was added directly to 15 ml of liquid scintillation fluid [1.05 g POPOP (Packard Instruments, Downers Grove, Ill.), 17.5 g PPO (Packard), 875 ml alcohol, and 2625 ml toluene (Fisher Scientific, Springfield, N.J.)] and counted in a refrigerated Beckman LS 250 liquid scintillation counter. (Corcino et al.\(^3\) added 0.5 ml of the autoclaved supernatent to 0.1 ml of 50% \(\text{H}_2\text{O}_2,\) solubilized the mixture in 4 ml of Hyamine, and then added 15 ml of scintillation fluid.) The Corcino et al.\(^3\) procedure was changed here because of the high quenching produced by Hyamine and \(\text{H}_2\text{O}_2\) Quenching was corrected for by using controls prepared by treating bone marrow without added label in identical manner to labeled cells. Counts per minute (cpm) ranged between 100 and 200 times background. All results were recorded as picograms folate uptake per \(10^6\) nucleated bone marrow cells. All of the vitamin B\(_12\)-deficient patients (all of whom had pernicious anemia) and the folate-deficient patient had megaloblastic bone marrows as shown morphologically and biochemically. The biochemical abnormality was documented by a "dU suppression test,"\(^4,5\) indicating overt megaloblastic DNA synthesis on the day of folate uptake study, and was further documented by correction of the "dU defect" by addition of B\(_12\) or PGA to the bone marrow suspension.\(^5\) Serum B\(_12\) and folate levels were determined by standard methods.\(^6,7\)

RESULTS

Figure 1 shows the uptake of \(^3\)H-PGA and \(^3\)H-MeTHF by bone marrow cells from the four hematologically normal patients as compared to three patients with pernicious anemia and the one patient with nutritional folate deficiency (serum folate = 1.5 ng/ml). (The pernicious anemia patients had serum B\(_12\) levels less than 10 pg/ml and serum folates above 7 ng/ml). In the normal patients and the folate-deficient patients, \(^3\)H-MeTHF uptake was about twice that of \(^3\)H-PGA. Conversely, bone marrow cells from each of the patients with pernicious anemia showed no significantly greater uptake of \(^3\)H-MeTHF than of \(^3\)H-PGA. (One actually showed the reverse—greater uptake of \(^3\)H-PGA than of \(^3\)H-MeTHF).

The B\(_12\)-deficient cells appeared to take up less of each folate per cell than did normal bone marrow cells. The folate-deficient marrow cells were like B\(_12\) deficient cells in that they appeared to take up less of each label than did normal cells. However, the folate-deficient cells were like normal cells in that they took up approximately twice as much \(^3\)H-MeTHF as they did of \(^3\)H-PGA (Fig. 1).

Figure 2 records the change in \(^3\)H-MeTHF uptake after 1-hr preincubation with 1 \(\mu\)g/ml of cyanocobalamin. Only the patients with pernicious anemia showed significant increase in \(^3\)H-MeTHF uptake after such preincubation. Not shown in the figure are the data demonstrating no significant increase in \(^3\)H-PGA uptake by either normal or B\(_12\)-deficient cells after preincubation with B\(_12\) (i.e., changes varied from \(-8\)% to \(+10\)%, which were not statistically significant).

DISCUSSION

The data demonstrating uptake of \(^3\)H-MeTHF twice that of \(^3\)H-PGA in normal and folate-deficient cells but not in B\(_12\)-deficient cells, plus the finding that \(^3\)H-MeTHF uptake by B\(_12\)-deficient cells is enhanced by added B\(_12\),
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Fig. 1. Uptake of ³H-PGA and ³H-MeTHF per 10⁶ nucleated bone marrow cells from normal vs. B₁₂-deficient (PA, pernicious anemia) vs. folate-deficient individuals. Range of triplicate determinations is shown protruding from top of each bar.

Fig. 2. Per cent change in uptake of ³H-MeTHF (per 10⁶ nucleated bone marrow cells) brought about by addition of B₁₂ to incubation medium. Baseline "zero value" is the uptake of ³H-MeTHF of control cells (i.e., in absence of added B₁₂). Range of triplicate determinations is shown protruding from top of each bar.

support the concepts that not only is cell uptake of ³H-MeTHF preferred over that of ³H-PGA, but also that cell uptake of MeTHF is B₁₂-dependent. Failure of added B₁₂ to correct completely defective folate uptake by B₁₂-deficient cells may relate, in part, to the short incubation time.

The unexpected finding of less than normal folate uptake by folate-deficient cells is analogous to the previously reported reduced B₁₂ uptake by B₁₂-deficient reticulocytes. The mechanisms of the two situations are unknown at present; it would be of interest to determine whether megaloblasts produced by purine or pyrimidine antagonists also have reduced B₁₂ or folate uptake.

The concept of B₁₂ dependence of cellular uptake of MeTHF is consistent with prior findings in patients with pernicious anemia. As reported a decade ago, serum folate activity in patients with pernicious anemia falls within 24 hr after the initiation of B₁₂ therapy. A clue to the present results was the finding that this fall is accompanied by an increase in red cell folate, to the high concentration of folate in the reticulocyte-rich blood after B₁₂ therapy, compared to the below-normal folate in reticulocyte-poor blood before therapy. A number of other workers have also observed that patients with pernicious anemia may have elevated serum folate activity and low red and white blood cell folate.
In B12 deficiency there appears to be inability to utilize folate adequately, which is "trapped" as MeTHF. MeTHF requires vitamin B12 to "untrap" it and allow it to get back into the usable folate pool as tetrahydrofolic acid.\textsuperscript{2,8,15-18} Strong support for the concept that methylfolate clearance from serum is dependent on B12 has recently been adduced by Nixon and Bertino.\textsuperscript{15}

The current study would tend to enlarge the "methylfolate trap" hypothesis to include a B12 requirement not only for intracellular utilization of methyl folate, but also for cellular uptake (and/or retention) of methyl folate. Such a hypothesis would offer an explanation for the low folate activity in B12 deficiency not only in blood cells,\textsuperscript{9-13} but also in liver cells.\textsuperscript{18-20} (It should be noted, however, that normal hepatic folate has been reported in B12 deficiency.\textsuperscript{21})

Failure of most prior studies to demonstrate reduced folate uptake by B12-deficient cells may relate to the finding reported here that this defect is primarily in uptake of methylfolate, and prior studies were conducted with PGA, whose cellular uptake in the present studies was not shown to be B12 dependent. It should be noted that Das and Hoffbrand did find impaired \textsuperscript{14}C uptake by B12-deficient lymphocytes incubated with \textsuperscript{14}CMeTHF, but from their data,\textsuperscript{22} it was not possible to determine whether the impairment was in uptake of \textsuperscript{14}CMeTHF or uptake of labile \textsuperscript{14}CMe groups no longer on THF. Since the radioactivity uptake they observed in B12-deficient lymphocytes incubated with \textsuperscript{14}CMeTHF was more than 250 times that which they observed with \textsuperscript{3}HPGA, we lean to the belief that they were measuring lymphocyte uptake primarily of labile methyl groups.

Whether the B12 dependence of cell uptake of methylfolate relates to methyltransferase (perhaps partly fixed to cell membrane) has not yet been determined. A cell membrane-fixed apoenzymelike binder for methylfolate could constitute a B12-dependent mechanism for cell uptake of methylfolate. Such a mechanism could deliver methylfolate or tetrahydrofolate in either direction (i.e., into or out from the cell).

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**REFERENCES**

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