

Impaired Utilization of Serum Folate in Pernicious Anemia

A STUDY WITH RADIOLABELED 5-METHYLTETRAHYDROFOLATE

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ABSTRACT The possible role of cobalamins in the utilization of serum methyltetrahydrofolate has been investigated by means of radiolabeled methyltetrahydrofolate in subjects suffering from pernicious anemia. After intravenous administration, methyltetrahydrofolate-³H (SA 11,500 Ci/mole; dose 0.05 µg/kg) was cleared from the serum to tissues of B₁₂-deficient subjects half as fast as after the same subjects had received vitamin B₁₂ therapy. B₁₂ deficiency was also associated with an increased rate of renal excretion of methyltetrahydrofolate or its derivatives, and a decreased rate of renal metabolism of methyltetrahydrofolate to other urinary folate derivatives.

Intravenously administered methyl-¹⁴C-tetrahydrofolate-³H at a higher dose (5 µg/kg) caused a severalfold elevation of the total serum folate concentration and, in B₁₂-deficient subjects, it did not disappear from the serum significantly more slowly although its urinary excretion was significantly increased.

These results indicate that there is some cobalamin requirement for the utilization of serum methyltetrahydrofolate and verify one prediction of the "methyltetrahydrofolate trap" explanation for the megaloblastosis of B₁₂ deficiency.

INTRODUCTION

The close relationship, which is apparent in hemopoiesis between vitamin B₁₂ and folic acid, appears to be the result of some role of cobalamins in the utilization of

folates (1), but is yet to be clearly defined. The best-documented hypothesis advanced to explain such a role for cobalamins in the utilization of folates is that of Noronha and Silverman (2) and Herbert and Zalusky (3) and is known as the "methyltetrahydrofolate trap" hypothesis. This hypothesis depends on the identification of both methylcobalamin and 5-methyltetrahydrofolate (meTHF)¹ as essential components for a single enzymic reaction, namely the biosynthesis of methionine from homocysteine (4-6) and on the finding that meTHF is normally the principal folate found in serum (3) and the principal monoglutamic folate found in liver (7, 8). The clinical effects of cobalamin deficiency are, with few exceptions, consistent with trapping of folates as meTHF and consequent intracellular operational deficiency of other folate coenzymes (1). One prediction of the methyltetrahydrofolate trap hypothesis which has not yet been verified is that, in cobalamin deficiency, there should be an expanded intracellular and total body pool of meTHF, or of its polyglutamates relative to other folates. This expanded meTHF pool should be demonstrable directly in the liver and indirectly as a slowing of the rate of transfer of meTHF from serum to tissues.

Previous investigations (9) have failed to demonstrate any slowing, in cobalamin deficiency, of the serum meTHF clearance rate after the administration of large doses of meTHF, sufficient to elevate the serum folate concentration severalfold. This paper reports data which demonstrate that the serum clearance of meTHF is measurably slowed in cobalamin deficiency, provided that the serum meTHF concentration is not elevated under the experimental conditions. For this purpose, tritiated meTHF of high specific radioactivity was administered in tracer doses to patients suffering from pernicious anemia, both before and after vitamin B₁₂ therapy.

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¹ Abbreviations used in this paper: meTHF, 5-methyltetrahydrofolate; meTHF-³H, 5-methyltetrahydrofolate-9,3',5'-³H; me-¹⁴C-THF, 5-methyl-¹⁴C-tetrahydrofolate; me-¹⁴C-THF-³H, methyl-¹⁴C-tetrahydrofolate-³H.

METHODS

General experimental procedures. Five male subjects were selected for study, each of whom suffered from a megaloblastic macrocytic anemia subsequently shown to be due to vitamin B₁₂ deficiency and pernicious anemia. Of the five subjects, three received intravenous injections of meTHF-³H at a dosage of 0.05 µg/kg body weight both before and subsequent to vitamin B₁₂ therapy. The other two received intravenous injections of me-¹⁴C-THF-³H at a dose of 5 µg/kg body weight before vitamin B₁₂ therapy. After administration of radiolabeled meTHF, serum and urine were collected at timed intervals and were counted for radioactivity, or were chromatographed to determine which chemical species of folate derivatives were radiolabeled. Serum concentration and urinary clearance of administered radiolabels were plotted as a function of time.

Materials. The physiological diastereoisomer of 5-methyltetrahydrofolate-9,3',5',-³H (meTHF-³H) and of 5-methyl-¹⁴C-tetrahydrofolate (me-¹⁴C-THF) was each prepared by described procedures (10) and stored frozen in the presence of 6 g/liter sodium ascorbate pH 6.0.

At the time of use, the radiochemical purities (10) of the preparations were 99 and 96% respectively. Preparations were diluted in normal saline, filter sterilized, and shown to be pyrogen-free before intravenous administration. The two preparations were combined in appropriate ratio for administration of me-¹⁴C-THF-³H. Dosage on a body weight basis was 0.05 µg/kg of meTHF-³H of specific radioactivity 11,500 Ci/mole or 5 µg/kg of me-¹⁴C-THF-³H of final specific radioactivities 33 Ci/mole (³H) and 13 Ci/mole (¹⁴C).

Radioactivity determinations. Of each of the following samples, 1 ml was mixed with 10 ml of a high efficiency dioxane-based scintillation mixture^a and counted in a liquid scintillation spectrometer: material for injection; serum deproteinized by perchloric acid; urine; and effluent fractions from analytical chromatographic columns. The results were corrected to disintegrations per minute by means of an external standard and comparison with experimentally-determined counting efficiency calibration curves. Samples containing both ¹⁴C and ³H were counted by scalars separately optimized for counting each radioisotope in the presence of the other; all scalars were optimized for the particular sample mixtures.

Chromatography. The identity of serum radiolabels was analyzed as follows. Collected serum was made 0.5 M in respect to 2-mercaptoethanol and stored frozen. After thawing, the solution which resulted from 40 ml of serum was expressed from the spongy protein precipitate and desalted by passage through a column of Sephadex G-15, 2.5 × 45 cm, together with 2 µmoles of each of the following marker compounds: meTHF, folic acid, and *p*-aminobenzoylethylglutamate. Elution was performed by 0.1 M potassium phosphate buffer pH 6.0 containing 20 mM 2-mercaptoethanol (starting buffer). Those fractions which contained radioactivity or marker compounds were pooled and applied to a column of A-25 DEAE-Sephadex 0.9 × 30 cm, equilibrated with starting buffer. A further marker, 2 µmoles of 5,10-methenylTHF, was applied and elution was achieved first by 40 ml of starting buffer in the mixing chamber and 250 ml of 2.0 M buffer (20 mM in 2-mercaptoethanol) in the reservoir. The collected fractions, each 2.5 ml, were monitored both for radioactivity, as described above, and for marker identity and concentration by spectra

recorded in the range 240–360 mµ using appropriate buffer as reference. The concentrations of either 10-formylTHF or 5,10-methenylTHF was monitored by absorbance measurements at 345 mµ after 1 hr incubation of the fractions at pH 1 and 37°C. This procedure converts any 10-formylTHF present to 5,10-methenylTHF. The use of this column allowed excellent resolution (11) of radiolabeled meTHF from other radiolabeled folate derivatives; the recovery of total applied radioactivity was greater than 90% from the G-15 column and greater than 97% from the A-25 column.

All urines were collected into bottles containing 2-mercaptoethanol to a final concentration of at least 0.2 M and were stored frozen. Thawed 20-ml portions of urine samples were chromatographed through G-15 and A-25 Sephadex similarly to serum.

Subjects. At the time of the initial administration of radiolabeled meTHF, each subject had a hematocrit in the range 22–26%, a macroovalocytosis, megaloblastic bone marrow, blood urea nitrogen 18–21 mg/100 ml, serum B₁₂ concentration 42–46 ng/liter and serum folate concentration 3.4–5.0 µg/liter. Subsequent gastric analyses and Schilling tests were compatible with the diagnosis of pernicious anemia, as were the hematological and general response to specific parenteral vitamin B₁₂ therapy. Before vitamin B₁₂ therapy, one subject, F. B., who also suffered from mild maturity-onset diabetes mellitus, showed early signs of posterolateral tract degeneration; another subject E. M. P., showed mild disorientation and memory loss.

The three subjects studied on two occasions each received their first injection of 1000 µg vitamin B₁₂ intramuscularly 24 hr after the first administration of 0.05 µg/kg meTHF-³H. Subjects C. G. and F. B. each received meTHF-³H twice by rapid intravenous injection, with intervals of 3 and 39 days, respectively, between doses. E. M. P. received meTHF-³H by intravenous infusion over a period of 2.5 hr, on two occasions at an interval of 39 days. At the time of their second study, F. B. and E. M. P. had hematocrits of 36% and respective serum vitamin B₁₂ concentrations of 340 and 360 ng/liter and serum folate concentrations of 10.4 and 9.4 µg/liter.

RESULTS

Serum chromatography. Serum collected from each B₁₂-deficient subject 3½–4 hr after the administration of meTHF-³H was chromatographed as described in Methods. A representative result is illustrated in Fig. 1. In each case, greater than 95% of the serum-³H was chromatographically identified with meTHF, indicating that the rate of disappearance of ³H from serum accurately measured the rate of disappearance of serum meTHF. In the case of subjects administered me-¹⁴C-THF-³H, the concentrations of radiolabels at 4 hr were insufficient for analysis, but a similar result was obtained at 1 hr.

Clearance rates for meTHF-³H. After the administration of meTHF-³H at the dosage of 0.05 µg/kg body weight, the peak total serum meTHF concentrations (obtained 2–5 min after injection) measured by *Lactobacillus casei* microbiological assay were not elevated above preinjection levels. These findings were consistent with the measured serum-³H peak concentra-

^a Aquafuor, New England Nuclear, Boston, Mass.

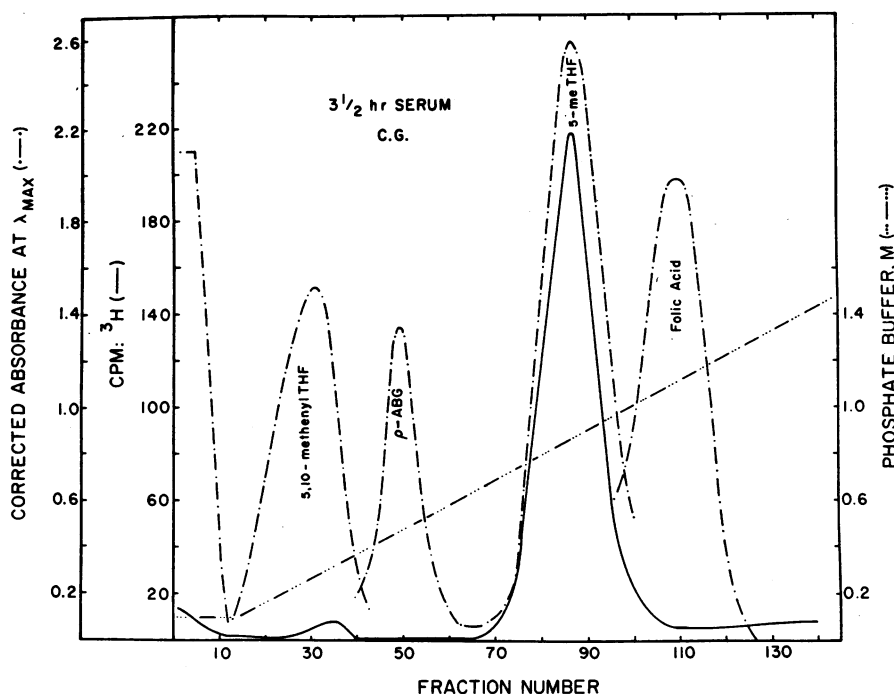


FIGURE 1 Serum chromatography. Profile of ^3H and markers eluted from DEAE-Sephadex for serum taken at 3.5 hr from C. G. The concentration and identity of marker compounds was determined as in Methods, and is shown as the absorbance due to each marker at the wavelength of maximum absorbance. The per cent recovery of the markers used was greater than 90% in all instances. The corrected absorbance for each compound at λ_{max} for the compound was obtained by subtracting the absorbance due to overlapping substances obtained as described in experimental.

tions which were equivalent to less meTHF than 0.5 $\mu\text{g/liter}$ in each case.

The serum disappearance rates for administered meTHF- ^3H were initially more rapid than exponential. The disappearance curve after rapid intravenous administration to subject F. B. is illustrated in Fig. 2A; that for C. G. was similar; that following constant infusion of the same total dose over a period of 2.5 hr to E. M. P. is shown in Fig. 2B. The serum disappearance rate for each B_{12} -deficient subject was slow compared with that determined later when the same subject was B_{12} replete.

At 24 hr after the administration of meTHF- ^3H , when the rates of disappearance of serum- ^3H were tending to become exponential, the subjects received 1000 μg of cyanocobalamin intramuscularly. Apparently, as a result of this vitamin B_{12} administration, the rates of disappearance of serum- ^3H were increased in the B_{12} -deficient subjects but not when the same subjects were already B_{12} replete (Fig. 2A and 2B).

After the intravenous administration of meTHF- ^3H , the cumulative urinary clearance of ^3H , illustrated in Fig. 3A and 3B followed a hyperbolic pattern. The total ^3H cleared by each subject in the first 5 hr was

not very different whether he was B_{12} deficient or B_{12} replete, but in the next period of 5–10 hr, at least twofold more tritium was cleared by the subjects F. B. and E. M. P. when they were B_{12} deficient than when they were B_{12} replete. There was no consistent immediate effect of vitamin B_{12} administration on the urinary clearance of ^3H , and there was no significant difference between the urinary excretion patterns for B_{12} deficiency and B_{12} repletion in the case of C. G., in whose case the repeat study was commenced only 2 days after the first administration of vitamin B_{12} . The cumulative urinary clearance of ^3H at 5 hr was less than 1% of the administered dose for F. B. and C. G., and 4% for E. M. P. At no time up to 48 hr in any subject did the cumulative urinary excretion of ^3H exceed 10% of the administered dose.

The urinary ^3H excretion data were subtracted from the serum- ^3H concentration data by use of urinary excretion rates determined by interpolation from each urine collection period, by use of values for the whole blood volume of 71 ml/kg body weight (12) and the ratio of total hematocrit to venous hematocrit of 0.9 (13) and by use of the measured venous hematocrit. The resultant corrected serum- ^3H concentration data

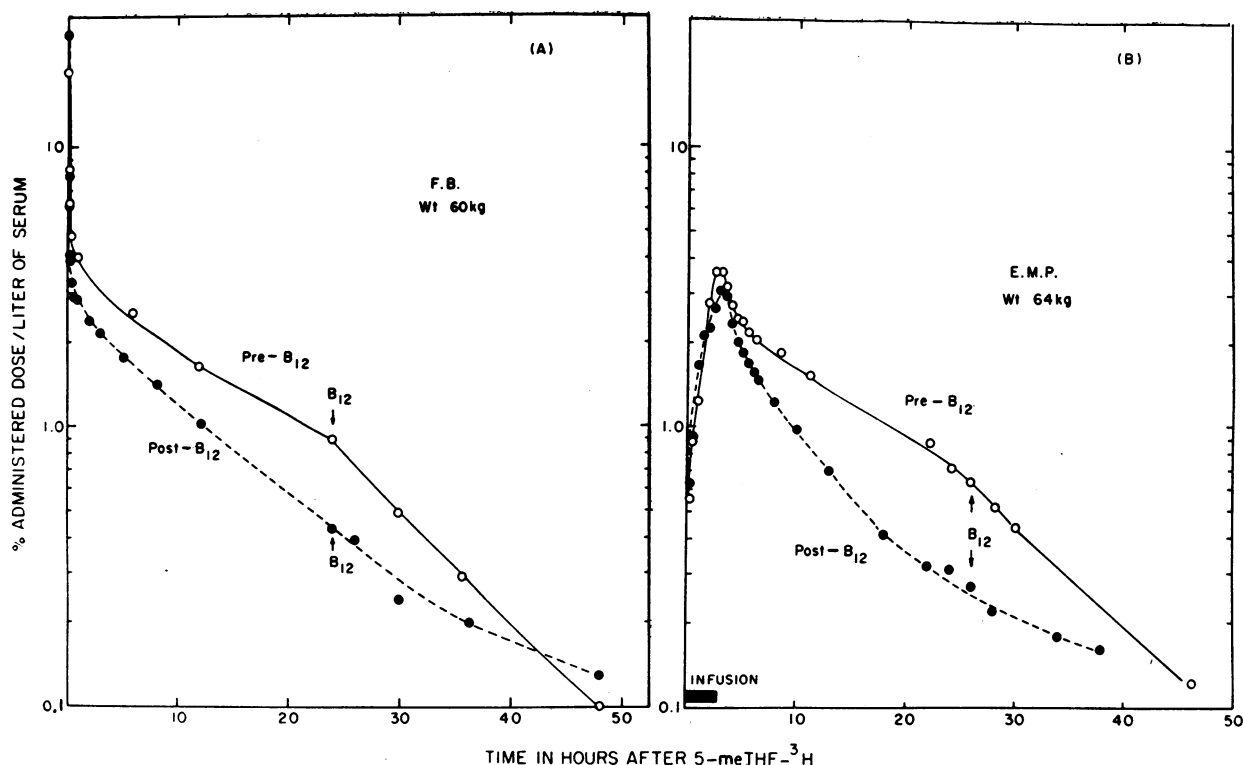


FIGURE 2 Rates of disappearance of serum meTHF- ^3H after intravenous administration rapidly (F. B.) and by constant infusion over 2.5 hr (E. M. P.) of a total dose $0.05 \mu\text{g/kg}$ body weight. Data for B_{12} -deficient subjects is shown, \bigcirc — \bigcirc ; data for repeat administration 39 days later when the subjects were B_{12} replete is shown \bullet — \bullet $1000 \mu\text{g}$ cyanocobalamin was administered intramuscularly at 24 hr in each study.

measured uptake of serum meTHF- ^3H into tissues apart from urinary clearance. Like the uncorrected data, the corrected serum meTHF- ^3H data did not provide straight-line regressions to first order disappearance plots, but did fit straight-line regressions when plotted as the inverse of serum meTHF- ^3H concentration against time; i.e., as second order plots. Representative resultant plots are illustrated in Fig. 4A and 4B. In each case, the second order rate constant for the clearance of serum meTHF- ^3H to tissues was significantly greater when the subject was B_{12} replete than when the same subject was B_{12} deficient. The second order rate constants, in units $\text{Ci}^{-1} \text{ liter hr}^{-1}$, for the three subjects when B_{12} deficient and B_{12} replete, respectively, were as follows: F. B., 0.05, 0.08; E. P., 0.06, 0.13 and C. G., 0.09, 0.20.

Clearance rates for me- ^{14}C -THF- ^3H . After the intravenous administration of me- ^{14}C -THF- ^3H at the dose $5 \mu\text{g/kg}$ body weight, meTHF levels measured in serum obtained at 3–5 min after the injection of *L. casei* were found to be elevated to over $60 \mu\text{g/liter}$. These values were consistent with the measured serum- ^3H peak con-

centrations which were equivalent to more meTHF than $50 \mu\text{g/liter}$ in each case.

The serum disappearance rates for ^3H and for ^{14}C were only insignificantly slower in the two B_{12} -deficient subjects than those observed in our laboratory for three B_{12} -replete subjects.³ However, the urinary excretion of radiolabels was twofold greater for ^3H and fourfold greater for ^{14}C than that of the controls, and the excretion of ^3H reached 15% of the administered dose within 2 hr and 28% within 6 hr. The excretion of ^{14}C reached 27% within 2 hr and 44% within 6 hr. The urinary excretion accounted for such a large proportion of the total disappearance of the radiolabels from serum that the rates of disappearance of radiolabels from serum were insensitive as a measure of uptake of meTHF from serum into tissues. This was true of all subjects studied by means of an intravenous dose of meTHF of $5 \mu\text{g/kg}$, irrespective of the condition of the subject or of the radiolabel used.

Urinary radiolabeled products. Chromatography of urine samples indicated that serum meTHF- ^3H was

³ Unpublished observations from our laboratory.

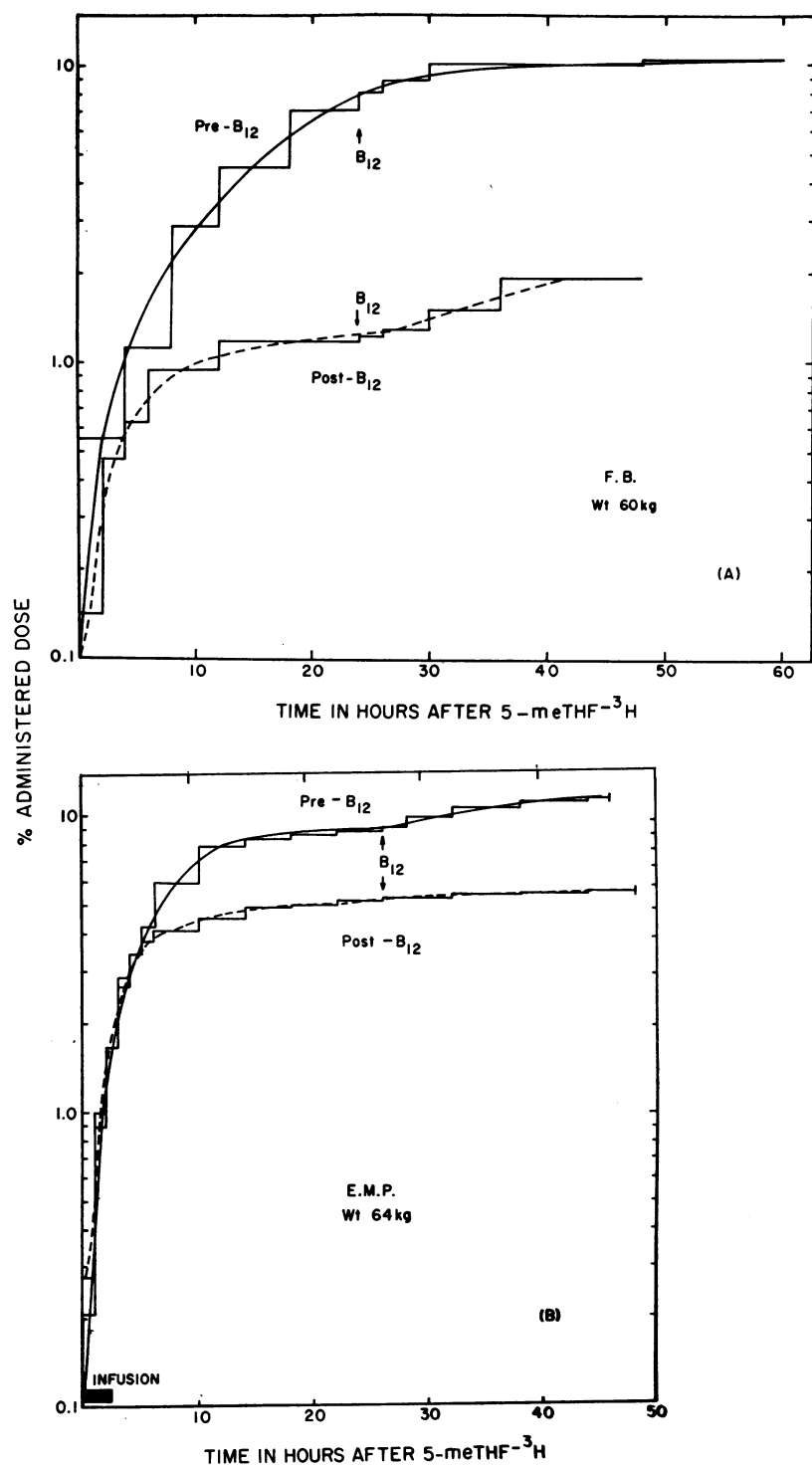


FIGURE 3 Cumulative urinary clearance of ^3H after intravenous administration of $5\text{-meTHF-}^3\text{H}$ rapidly (F. B.) and by constant infusion over 2.5 hr (E. M. P.).

extensively metabolized during renal excretion (Fig. 5A and 5B). In every instance, less than 40% of the urinary ^3H was identified as meTHF- ^3H . The remaining chromatographically-separable peaks of ^3H included one which eluted late, about the position of dihydrofolate or folate, and a large complex peak overlapping the

known elution position (11) of 5,10-methenylTHF (or 10-formylTHF), pteridines, and *p*-aminobenzoylglutamate. The urines of subjects administered me- ^{14}C -THF- ^3H also contained a ^{14}C peak, in the elution position of 5,10-methenylTHF. Generally, the proportion of the total urinary ^3H which could be identified as meTHF-

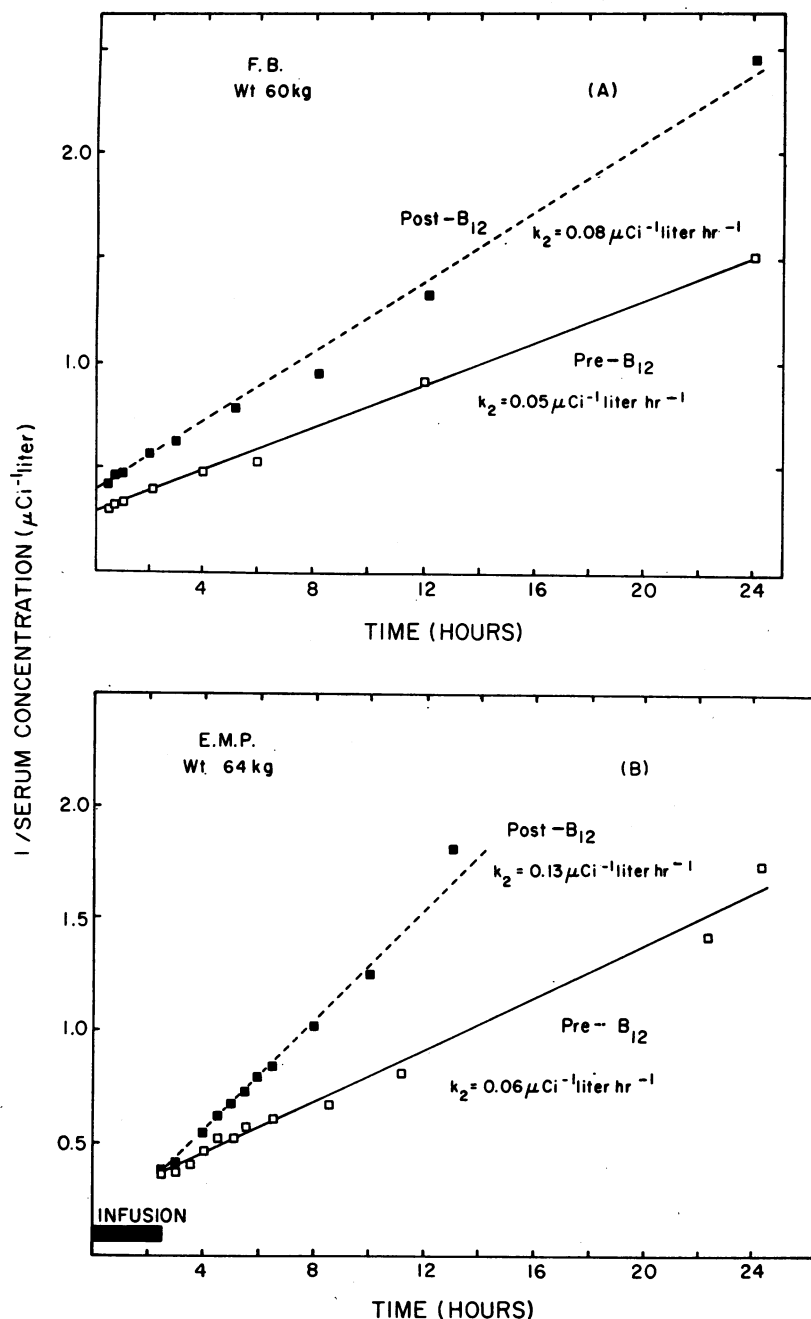


FIGURE 4 Second order rate plots for data shown in Fig. 2, corrected as described in Results. The straight regression lines were fitted by the least squares method.

^3H was greater than 30% in the 0-4 hr time period and progressively decreased to about 15-16% in the 24-48 hr time period. In the interval 4-12 hr, the proportion of total urinary ^3H identifiable as meTHF-

^3H was 36-38% for each B_{12} -deficient subject but only 27-28% for the same time period when the subject was B_{12} replete. The results of chromatograms representing this time period for one subject, C. G., are illustrated

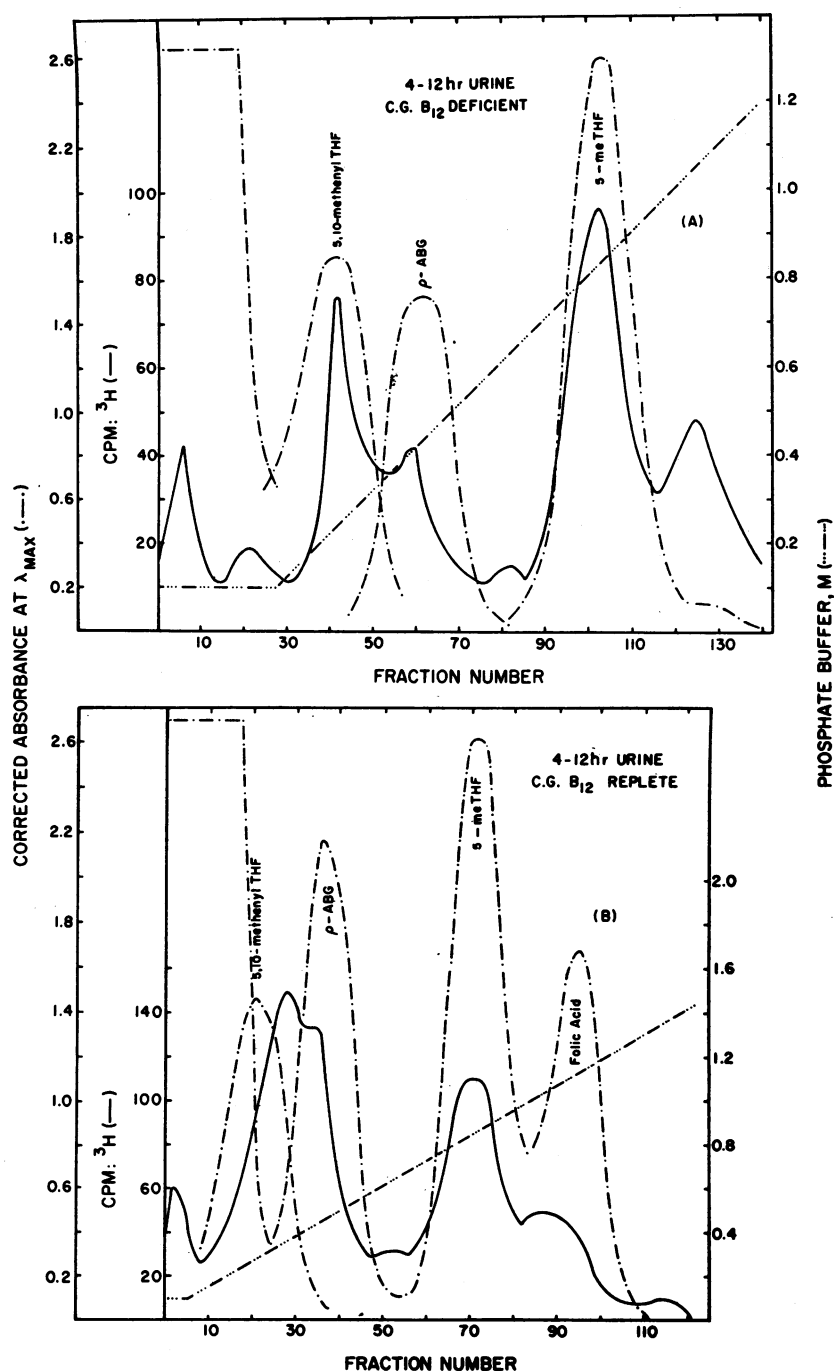


FIGURE 5 Urine chromatography. Profile of ^3H and markers eluted from DEAE-Sephadex, for portions of urines collected from C. G. in the time period 4-12 hr; Fig. 5A when B_{12} deficient, Fig. 5B when B_{12} replete.

in Fig. 5A and 5B. In other time periods the proportion of total ^3H which was excreted as meTHF- ^3H appeared to be possibly dependent on the subject's vitamin B $_{12}$ status in a similar manner, but the differences were quantitatively insignificant.

DISCUSSION

The results of experiments using high specific radioactivity meTHF- ^3H at the very low dose of 0.05 $\mu\text{g/kg}$ clearly demonstrated a dependence on cobalamins for the clearance of meTHF from serum to tissues. The use of me- ^{14}C -THF- ^3H might have allowed additional measurement of the rate of methyl group disappearance but, the necessary dosage of me- ^{14}C -THF- ^3H was governed by the requirement for measurable concentrations of radiolabels in the serum and the relatively low maximum specific radioactivity of ^{14}C . Thus, the use of me- ^{14}C -THF- ^3H required total doses of 5 $\mu\text{g/kg}$, considerably larger than tracer doses. At this dosage the initial serum total meTHF concentration was elevated severalfold and the renal clearance of meTHF and its derivatives was markedly increased. Under these conditions, a serum disappearance rate, even corrected for renal clearance, could not be relied on as an accurate measure of differences in the rate of transfer of meTHF from serum to other tissues. The results of experiments in which radiolabeled meTHF was used at 5 $\mu\text{g/kg}$ also explain the failure of other investigators (9) to detect any slowing of serum meTHF disappearance in B $_{12}$ deficiency, since in those investigations meTHF was administered only at 5 $\mu\text{g/kg}$.

The observed fit of the serum meTHF disappearance data to a second order regression suggests that meTHF disappearance from the serum is dependent on one or more factors additional to the serum concentration of meTHF. The nature of these factors is uncertain but may involve specific transport factors in serum or factors for cellular uptake or utilization or the operation of a number of body compartments additional to the three considered under Results; namely serum, renal tract, and all other tissues together. It is possible that these additional factors may relate to the liver, which accumulates folates to a total concentration 1000-fold higher than that of serum. The results do not permit the conclusion that these factors relate only to the cobalamins, although that is possible.

The results of chromatography of urines demonstrated an extensive metabolism of folates during their transfer from serum to urine. The nature of those changes is uncertain but a particularly interesting observation was the occurrence of material which was labeled with both ^{14}C and ^3H , in experiments in which me- ^{14}C -THF- ^3H was administered, and which eluted

from DEAE-Sephadex in the position of 10-formylTHF or 5,10-methenylTHF. Similar urinary material has been observed in our laboratory after the oral or intravenous administration of 5-formyl- ^{14}C -THF- ^3H (14). Whatever the nature of the renal metabolism of meTHF, it was apparently decreased in cobalamin deficiency. Alternatively, the accumulation of metabolic products of meTHF in the urine in concentrations not observed in the serum could represent less efficient renal conservation of these compounds. Some evidence exists for less effective tubular reabsorption of 5-formylTHF than meTHF (14).

The results demonstrate that the normal tissue utilization of serum meTHF does require a cobalamin. The observed decreased rate of transfer of meTHF from serum to tissues was only twofold, but this may have been the result of a much larger intracellular decrease in meTHF utilization in the liver. Certainly the results described are consistent with the "meTHF trap" hypothesis and verify, for the first time, one further prediction (1) of that hypothesis (2, 3), i.e., that normal tissue utilization of serum meTHF does require a cobalamin. At this stage a meTHF trap appears increasingly attractive as an explanation for the megaloblastosis of vitamin B $_{12}$ deficiency. Firm establishment of a meTHF trap might require measurement of a decreased meTHF methyl group turnover in the liver and seems hardly justifiable in patients suffering from pernicious anemia, since it would require multiple liver biopsies.

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REFERENCES

1. Nixon, P. F., and J. R. Bertino. 1970. Interrelationships of vitamin B $_{12}$ and folate in man. *Amer. J. Med.* **48**: 555.
2. Noronha, J. M., and M. Silverman. 1962. On folic acid, vitamin B $_{12}$, methionine and formiminoglutamic acid, metabolism. *Eur. Symp. Vitam. B $_{12}$ Intrinsic Factor 2nd*, 1961. 728.
3. Herbert, V., and R. Zalusky. 1962. Interrelations of vitamin B $_{12}$ and folic acid metabolism: folic acid clearance studies. *J. Clin. Invest.* **41**: 1263.
4. Hatch, F. T., A. R. Larrabee, R. E. Cathou, and J. M. Buchanan. 1961. Enzymatic synthesis of the methyl group of methionine. I. Identification of the enzymes and cofactors involved in the system isolated from *Escherichia coli*. *J. Biol. Chem.* **236**: 1095.
5. Foster, M. A., K. M. Jones, and D. D. Woods. 1961. The purification and properties of a factor containing vitamin B $_{12}$ concerned in the synthesis of methionine by *Escherichia coli*. *Biochem. J.* **80**: 519.

6. Loughlin, R. E., H. L. Elford, and J. M. Buchanan. Enzymatic synthesis of the methyl group of methionine. VII. Isolation of a cobalamin-containing transmethylese (5-methyltetrahydrofolate-homocysteine) from mammalian liver. *J. Biol. Chem.* **239**: 2888.
7. Donaldson, K. O., and J. C. Keresztesy. 1961 Further evidence on the nature of Prefolic A. *Biochem. Biophys. Res. Commun.* **5**: 289.
8. Noronha, J. M., and M. Silverman. 1962. Distribution of folic acid derivatives in natural materials. I. Chicken liver folates. *J. Biol. Chem.* **237**: 3299.
9. Chanarin, I., and J. Perry. 1968. Metabolism of 5-methyltetrahydrofolate in pernicious anemia. *Brit. J. Haematol.* **14**: 297.
10. Nixon, P. F., and J. R. Bertino. 1971. Enzymic preparations of radiolabeled (+), L-5-methyltetrahydrofolate and (+), L-5-formyltetrahydrofolate. *Anal. Biochem.* **43**: 162.
11. Nixon, P. F., and J. R. Bertino. 1971. Separation and identification of folate coenzymes on DEAE-Sephadex. In *Vitamins and Coenzymes. Methods Enzymol.* **18B**: 661.
12. Gray, J. J., and H. Frank. 1953. The simultaneous determination of red cell mass and plasma volume in man with radioactive sodium chromate and chromic chloride. *J. Clin. Invest.* **32**: 1000.
13. Chaplin, H., P. L. Mollison, and H. Vetter. 1953. The body/venous hematocrit ratio: its constancy over a wide hematocrit range. *J. Clin. Invest.* **32**: 1309.
14. Nixon, P. F., and J. R. Bertino. 1972. Effective absorption and utilization of oral formyltetrahydrofolate in man. *N. Engl. J. Med.* **286**: 175.