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Effect of Methionine on Hepatic Folate Metabolism in Rats Fed a Vitamin B12- and Methionine-Deficient Diet[1] (39823)

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Introduction. The similarities in the clinical and biochemical symptoms of vitamin B12 deficiency and folate deficiency in man and in experimental animals have been reviewed by several investigators (1-3). Functional deficiency of folate in rats does not occur with vitamin B12 deficiency unless this deficiency is accompanied by a low-methionine content in the diet (4, 5). One theory for the interrelationship between vitamin B12 and folate metabolism, advanced by Noronha and Silverman (6) and by Herbert and Zalusky (7) is the "methyl trap" hypothesis. This theory suggests that 5-methyl-H_4PteGlu[3] can only be converted to other folate coenzymes via the B12-dependent 5-methyltetrahydrofolate-homocysteine transmethylase reaction (8) and, under conditions of vitamin B12 deprivation, the activity of this enzyme is significantly reduced (9); as a result, a large amount of folate is "trapped" as the methyl derivative at the expense of other folate coenzymes. This hypothesis is strengthened by the observation that the methylenetetrahydrofolate reductase reaction, in which 5-methyl-H_4PteGlu is formed, is essentially irreversible under physiological conditions (10) and that *S*-adenosylmethionine is a noncompetitive inhibitor of the reductase (10). Methionine therefore can prevent folate from being trapped as 5-methyl-H_4PteGlu under conditions of vitamin B12 deficiency by feedback control over the synthesis of 5-methyl-H_4PteGlu. Experimentally, methionine supplementation of the vitamin B12- and methionine-deficient rat, either in the diet or by injection, doubled the level of liver AdoMet (11), decreased the urinary excretion of formiminoglutamate (FIGlu) and formate (12), increased the metabolism of histidine and formate (12), and increased the content of liver folate (13).

Recently, a second theory for the interrelationship between vitamin B12 and folate metabolism has been advanced; that methionine and vitamin B12 are involved in the membrane transport of folates (14, 15).

Because of recent advances in techniques for the identification of folate coenzymes in the rat liver (16-19, 22), we undertook the present research to reevaluate the effect of methionine on the metabolism of labeled folate in the vitamin B12- and methionine-deficient rat. The results are discussed in relation to the "methyl trap" and transport theories for vitamin B12/folate interrelationships.

Materials and methods. Radioactive compounds. [^{3}H]PteGlu (sp act 37 Ci/mmole) labeled at the 3',5', and 9 positions and 5-[^{14}C]methyl-H_4PteGlu (sp act 54 mCi/mmole) were purchased from Amersham/Searle Corp., Des Plaines, Ill. Both compounds were purified by QAE-Sephadex chromatography (20) before use.

Animals, diets, and tissue preparation. Male, weanling Sprague-Dawley rats weighing 50-70 g were housed in a light- and temperature-controlled room with food and water supplied ad libitum.

The 20% soy-protein, vitamin-B12-free, methionine-low (0.2%) diet was prepared as described previously (12). Animals were maintained on the diet for 10-12 weeks, by which time they had become vitamin deficient as evidenced by an elevated FIGlu excretion. Animals were then injected intraperitoneally with 15 μCi of [^{3}H]PteGlu (in 0.3 ml of saline). In some experiments, 250 μmole of L-methionine (37 mg/0.5 ml of saline) was injected 15 min preceding the [^{3}H]PteGlu injection. At various times after

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[3] Abbreviations used: PteGlu, pteroylglutamic acid; AdoMet, *S*-adenosylmethionine.

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the injection, rats were killed by decapitation and bled for 30 sec. Livers were removed and aliquots (5 g) were cut into thin slices which were then dropped into 20 ml of 1.1% ascorbate, pH 6.0, maintained at 95°. After heating for 10 min to inactivate γ-glutamyl carboxypeptidase, the mixtures were homogenized and centrifuged and the supernatants were stored at −20° until used. The remainder of each liver was homogenized in 6.5 vol of 0.31 *M* trichloroacetic acid containing 5 *M* urea, centrifuged, and the supernatant was collected for the determination of folate glutamate chain length.

DEAE-cellulose and Sephadex G-25 chromatography of liver folates. Folate compounds were chromatographed on DEAE-cellulose as described by Shin *et al.* (16). Ascorbate extract (5 ml; equivalent to 1 g of liver) containing added standard PteGlu (200 μg) and 5-[^{14}C]methyl-H_4PteGlu (8000 dpm) as markers was applied to a 25 × 0.9-cm DEAE-cellulose column. Folate derivatives were eluted by an exponential phosphate gradient formed with 50 ml of 0.01 *M* K-phosphate buffer, pH 6, in a closed mixing chamber attached to a reservoir containing 0.6 *M* K-phosphate buffer, pH 6. Both buffers contained 0.2 *M* 2-mercaptoethanol. One hundred twenty 2.5-ml fractions were collected.

Folate polyglutamates were also separated from mono- and diglutamates by chromatography on 190 × 0.9-cm Sephadex G-25 columns as described by Shin *et al.* (18). Ascorbate extract (2 ml; equivalent to 0.4 g of liver) was applied to each column and the columns were eluted with 0.1 *M* K-phosphate buffer, pH 7, containing 0.2 *M* mercaptoethanol. Eighty 1.7-ml fractions were collected.

Estimation of glutamate chain length of folates following cleavage to p-aminobenzoylglutamate (n) derivatives. This was carried out using the reductive cleavage method of Baugh *et al.* (17) as modified by Tyerman *et al.* (19) to include a subsequent purification of the *p*-aminobenzoylglutamate derivatives by adsorption on and elution from activated charcoal. This adsorption and elution serves to remove electrolytes so that the *p*-aminobenzoylglutamate extract can be applied to a DEAE-cellulose column in a relatively small volume (ca. 20 ml) of conductivity less than 1 mmho. An aliquot, equivalent to 2 g of liver, was applied to a 0.9 × 20-cm DEAE-cellulose (Whatman DE-52) column. This was eluted by an exponential salt gradient made up with 500 ml of 5 m*M* potassium phosphate, pH 7, in a closed mixing chamber attached to a reservoir containing the same buffer plus 0.5 *M* potassium chloride. One hundred twenty 4-ml fractions were collected.

Radioactive compounds in column fractions were determined by scintillation counting. Aliquots (1 ml) of the fractions were mixed with a Triton X-100:toluene (1:2) cocktail (10 ml) (21), and counting efficiencies were determined by an external standard method.

Results. Table I shows the effect of methionine on the hepatic uptake of labeled folate and on the distribution of labeled *p*-aminobenzoylpoly-γ-glutamates derived from labeled hepatic folates at various times

TABLE I. UPTAKE OF [^{3}H]PteGlu BY RAT LIVER AND ITS INCORPORATION INTO PTEROYLPOLY-γ-GLUTAMATE FORMS WITH TIME AS DETERMINED BY THE REDUCTIVE CLEAVAGE METHOD AND BY THE SEPHADEX G-25 CHROMATOGRAPHIC PROCEDURE.[a]

			Reductive cleavage method (percentage of hepatic uptake)							Sephadex G-25 (percentage of hepatic uptake)		
Time (hr)	Met[b] injection (37 mg)	Total hepatic uptake (% dose)	pABA-Glu_1	pABA-Glu_2	pABA-Glu_3	pABA-Glu_4	pABA-Glu_5	pABA-Glu_6	pABA-Glu_7	Folate (Glu_1) fr. 45-60	Folate (Glu_{2-3}) fr. 21-44	Folate (Glu_{4-7}) fr. 1-20
2	−	8	84	4	3	3	6	0	0	87	<5	13
4	−	9	71	16	6	3	3	0	0	94	<5	6
24	−	7	19	3	13	6	42	13	5	20	16	64
2	+	10	69	2	3	6	21	0	0	69	<5	31
4	+	18	61	6	6	10	11	4	2	74	<5	26
24[c]	+	21	3	1	6	11	42	34	2	6	15	79

[a] All rats were maintained on the vitamin B12- and methionine-deficient soy diet. Experimental details are described in the methods section.
[b] Methionine administered 15 min prior to [^{3}H]PteGlu injection.
[c] Methionine injected every 6 hr.

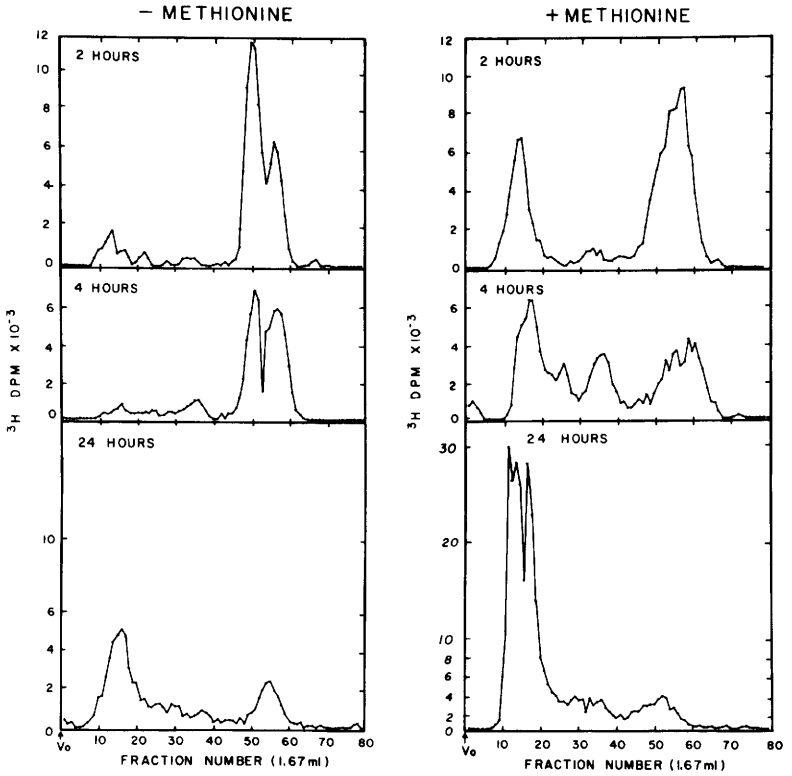

FIG. 1. Sephadex G-25 elution profiles of labeled hepatic folates at various times after intraperitoneal administration of [^{3}H]PteGlu to the vitamin B12- and methionine-deficient rat. Experimental procedures are described in the methods section. In some cases (+Methionine), rats were preinjected with methionine (37 mg) 15 min prior to the [^{3}H]PteGlu dose. The methionine injection was repeated every 6 hr for the 24-hr sample. The approximate elution positions of folate standards chromatographed under identical conditions were: PteGlu (fraction 58), $PteGlu_2$ (36), $PteGlu_3$ (28), $PteGlu_4$ (20), $PteGlu_5$ (16), $PteGlu_6$ (13), and $PteGlu_7$ (10).

after injection of [^{3}H]PteGlu to the vitamin B12- and methionine-deficient rat. At the time intervals studied, relatively small amounts of folate di-, tri-, and tetraglutamates were detected (<20%). Labeled liver folates were predominantly in the mono-, penta-, and hexaglutamate forms, indicating that synthesis of folate polyglutamates proceeds rapidly until the pentaglutamate is formed. Table I also shows the distribution of folate polyglutamates as determined by Sephadex G-25 chromatography (Fig. 1). Although the resolution of individual pteroylpolyglutamate forms obtained with this method is much poorer than that obtained with the reductive cleavage procedure, the results obtained with both methods are in close agreement.

About 8% of the labeled dose was taken up by the livers of the deficient animals, and the net uptake remained at about this level for 24 hr. A slow conversion of pteroylmonoglutamate to polyglutamate forms, predominantly the pentaglutamate, was observed. Methionine supplementation had little effect on the net uptake of the labeled folate dose at 2 hr, but did increase the net uptake considerably at 4 and 24 hr. This increased uptake by the methionine-supplemented animals was accompanied by an increased synthesis of pteroylpolyglutamates. The absolute amounts of labeled monoglutamate in the livers of the deficient and supplemented animals was similar 24 hr after the dose. Approximately 85% of the labeled folate in the livers of the deficient animals was in the 5-methyltetrahydrofolate form. Methionine supplementation decreased the proportion of methyltetrahydrofolate to approximately 55% of the total

labeled folate present.

Dietary methionine supplementation also decreased the proportion of methyltetrahydrofolate, increased the hepatic uptake of a labeled folate dose, and increased the synthesis of labeled pteroylpolyglutamates, but not as effectively as methionine administered by injection.

Discussion. In this study, the effect of methionine preinjection on the hepatic metabolism of a labeled folate dose in the vitamin B12- and methionine-deficient rat was investigated. Methionine caused an increase in the net hepatic uptake of labeled folate, decreased the proportion of 5-methyltetrahydrofolate, and caused a large increase in the hepatic levels of pteroylpolyglutamates. These data are consistent with the methyl trap hypothesis for vitamin B12/folate interrelationship and with prevention of the 5-methyl-H_4PteGlu trap by AdoMet inhibition of the methylenetetrahydrofolate reductase reaction.

This stimulation of pteroylpoly-γ-glutamate formation upon methionine supplementation was also reported by Buehring *et al.* (23) in the isolated perfused liver. The properties of the pteroylpoly-γ-glutamate synthetase are not well defined at the moment. However, preliminary results from our laboratory have shown that 5-methyl-H_4PteGlu is a poor substrate for the rat liver polyglutamate synthetase, whereas H_4-PteGlu is an efficient substrate for this enzyme (unpublished results). In vitamin B12 deficiency, the buildup of 5-methyl-H_4PteGlu would lower the rate of pteroylpoly-γ-glutamate synthesis and, as these are the forms of folate that are preferentially retained by tissues (24–26), a folate deficiency would ensue due to a failure to retain folates. This effect can be prevented by methionine supplementation inhibiting the formation of methyltetrahydrofolate which would generate more tetrahydrofolate, the substrate for the pteroylpolyglutamate synthetase.

The increased net uptake of labeled folate by the livers of methionine-supplemented animals is best explained by an increased synthesis of labeled pteroylpolyglutamates and a consequent increased retention of folates, rather than by methionine having a direct effect on the membrane transport of folates. It should be noted that methionine supplementation did not affect the net uptake of labeled folate in the first 2 hr, a period in which little synthesis of pteroylpolyglutamates occurred. A similar effect was recently reported in studies on the effects of vitamin B12 supplementation to the vitamin B12- and methionine-deficient rat (27).

Summary. The effect of methionine on the metabolism of hepatic folate in the vitamin B12- and methionine-deficient rat was investigated. The liver uptake of a labeled dose of PteGlu by the deficient rats remained constant at approximately 8% between 2 and 24 hr after the injection. At the early time periods (2 and 4 hr), the major labeled folate derivative was 5-methyl-H_4PteGlu and no appreciable synthesis of labeled pteroylpoly-γ-glutamate was detected. An injection of methionine (37 mg) suppressed the level of 5-methyl-H_4PteGlu and increased the synthesis of pteroylpoly-γ-glutamates. Methionine also increased the total net hepatic uptake of the labeled folate dose. The increased net uptake was entirely due to increased synthesis of pteroylpolyglutamates. These results are best explained by the methyl trap hypothesis for B12/folate interrelationships.

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