The Effect of Vitamin B_{12} and Methionine on Folic Acid Uptake by Rat Liver (35188)

J. M. GAWTHORNE[1] AND E. L. R. STOKSTAD

Department of Nutritional Sicences, University of California, Berkeley, California 94720

Changes in tissue concentrations of folate derivatives during vitamin B_{12} deficiency may have important consequences for 'one-carbon' metabolism. The liver is of special interest because this organ contains a major part of the total body folate, together with many folate-dependent enzymes.

While some researchers claim that liver folate levels are increased by vitamin B_{12} deficiency (1), others report no change (2), or a decrease (3, 4). The disparity in these reports is partly due to differences in assay technique. Bird *et al.* (5) illustrated the inadequacies of earlier preparatory techniques of folate assay, and outlined new methods using hog kidney and chicken pancreas conjugases that enabled consistent estimation of the entire folate content of tissues. These methods were used in the experiments of Kutzback *et al.* (4) but not in the other studies.

The disagreement between previous reports prompted us to reexamine the effect of vitamin B_{12} deficiency on liver folate levels in rats. The modifying influence of methionine (4), has been further investigated. By using radioactive folic acid it has been possible to observe the short-term effects of vitamin B_{12} and methionine supplements on net uptake of folic acid by liver *in vivo*.

Materials and Methods. Two experiments were performed. The first contained 40 animals in an investigation of the effect of dietary vitamin B_{12} and methionine on liver ^3H folic acid uptake, plasma folate concentration, and total liver folate levels. The second experiment, with 56 animals, was an examination of the effect of short-term intraperitoneal supplements of vitamin B_{12} and methionine on liver ^3H folic acid uptake.

The basal diet, animal housing, and technique for estimating ^3H folic acid uptake were similar in both experiments.

Diets. The ingredients of the basal diet were as follows (g/kg): soy assay protein, 200; glucose monohydrate, 714; corn oil with vitamins A, D, and E, 40; salt mix (6), 35; vitamin pre-mix in glucose monohydrate, 10; choline chloride, 1. The vitamins were supplied in the following amounts per kg: vitamin A, 15,000 IU; vitamin D (viosterol), 2000 units; α-tocopherol acetate, 50 mg; pyridoxine HCl, 15 mg; Ca-pantothenate, 50 mg; niacin HCl, 50 mg; menadione, 10 mg; folic acid, 5 mg. The basal diet was deficient in vitamin B_{12}, and contained only 0.2% methionine. When the basal diet was supplemented with vitamin B_{12} and/or *d,l*-methionine, the levels were 100 μg/kg and 7 g/kg, respectively.

Animals. Male, weanling Sprague-Dawley rats weighing 50-70 g were individually housed in stainless steel screen-bottom metabolism cages in a constant temperature room. In both experiments eight animals were randomly assigned to each of the experimental treatments. After 13 weeks, animals fed the basal diet were definitely vitamin B_{12}–methionine deficient on the basis of an excretion of methylmalonic acid of 29.0 μmoles/100 g/24 hr compared with 3.2 μmoles/100 g/24 hr in vitamin B_{12} supplemented animals.

The mean body weight of deficient animals was 440 g while vitamin B_{12} and methionine supplemented animals averaged 496 g. Measurement of the effect of dietary or intraperitoneal supplements of vitamin B_{12} and/or methionine on ^3H folic acid uptake, plasma folate concentration, or total liver folate levels were made during weeks 13–16 in each experiment. *^3H folic acid uptake.* Folic acid-

[1] CSIRO Post-doctoral Fellow. Present address: CSIRO, Division of Nutritional Biochemistry, Kintore Ave., Adelaide, South Australia, 5000.

3′,5′-T (potassium salt), 48.5 Ci/mmole was obtained from Amersham/Searle Corp. Just before use the purity of each batch was checked by DEAE chromatography. Batches of less than 90% purity were rejected. The radioactive folic acid was dissolved in 0.9% sodium chloride for injection, and most solutions contained between 2.5×10^5 and 3.0×10^5 dpm/μl. Each animal was given 2 μCi of ^{3}H folic acid/100 g of body weight by injection into a femoral vein. The animals were returned to their cages and permitted access to diet and water *ad libitum* for 10 hr. Exactly 10 hr after the administration of ^{3}H folic acid, animals were anesthetised and killed by removal of blood via heart puncture. The liver was flushed free of residual blood with a copious infusion of ice-cold 0.9% sodium chloride, then it was removed, blotted dry, and weighed. Two g of liver were homogenized and made up to 10 ml with cold 0.9% sodium chloride. Two 0.6-ml aliquots were placed in separate scintillation vials, 5 ml of 0.24 *N* NCS reagent (Nuclear Chicago Corp.) was added to each, and the samples were thoroughly mixed and incubated overnight at 45°. 10 ml of Bray's solution (9 mg of PPO/ml) were then added, and 24 hr later the radioactivity in the samples was counted in a Beckman scintillation counter. Adjustment for quenching was made by the Automatic External Standardization method. The counting efficiency in most samples was 19–23%. The total radioactivity in each liver was calculated, and the net uptake of ^{3}H folic acid was expressed as a percentage of the injected dose per 10 hr.

Liver and plasma folate assays. Immediately after weighing each flushed liver, a 1-g sample was removed, cut into small pieces with scissors, and dropped into 20 ml of 1% sodium ascorbate, pH 6.0, at 95°. Extraction of liver folates was continued at this temperature for 10 min, after which the extract was cooled in ice, homogenized, and centrifuged. The supernatant was stored frozen until assayed. Ascorbate extracts of liver were treated with hog kidney conjugase (5) before microbiological assay with *Lactobacillus casei.*

Heparinized blood was centrifuged, and 1.8 ml of plasma were added to 0.2 ml of 10% sodium ascorbate pH 6.0 in a glass tube. After mixing, the plasma was stored frozen until assayed with *L. casei.*

TABLE I. The Effect of Dietary Supplements of Vitamin B_{12} and Methionine on Uptake of Intravenously Administered ^{3}H Folic Acid by Rat Liver.

Diet fed 13 weeks	Dietary supplement 30 hr	^{3}H Folate uptake[e] (% in 10 hr)	Relative uptake (%)
Control[b]	—	17.2 ± 0.7^f	100
Basal[a]	—	$9.5 \pm 1.2^{g,h}$	55
	B_{12}[c]	7.4 ± 0.6^g	43
	Me[d]	13.0 ± 1.8^h	76
	B_{12} + Me	20.3 ± 0.6^f	118

[a] $-B_{12}$, —Me diet: deficient in Vitamin B_{12}, only 0.2% methionine.

[b] $+B_{12}$, +Me diet: basal diet with 100 μg of B_{12}/kg and 0.7% methionine added.

[c] 100 μg of B_{12}/kg.

[d] 0.7% methionine.

[e] Values are mean ± SE for eight animals. Means with different letters are significantly different ($p < 0.01$).

Statistics. The significance of differences between treatment means was evaluated by the Student's *t* test (7).

Results. Expt. 1. The net liver uptake of ^{3}H folic acid in animals fed the unsupplemented basal diet ($-B_{12}$, —Me) was only 55% of that in animals given the $+B_{12}$, +Me diet (Table I). Supplementation of the basal diet for 30 hr with 100 μg of B_{12}/kg, or 0.7% methionine did not significantly alter the folate uptake of deficient animals, but when vitamin B_{12} was given together with methionine the net folate uptake was increased to control values ($+B_{12}$, +Me, diet).

Concomitant changes in total liver and plasma folate levels are shown in Table II. Liver folate concentrations were greatly decreased by feeding the $-B_{12}$, —Me diet for 13 weeks, while plasma folate levels were elevated. Dietary supplementation with 100 μg of B_{12}/kg or 0.7% methionine for 30 hr did not significantly alter liver folate concentrations, but when rats were fed the vitamin B_{12} and methionine supplements together for 30 hr, liver folate levels increased, while plasma folate concentrations were depressed. The rise in liver folate levels represented an av-

TABLE II. The Effect of Dietary Supplements of Vitamin B_{12} and Methionine on Liver and Plasma Folate Levels.

Diet fed 13 weeks	Dietary supplement 30 hr	Liver wt[e] (g)	Liver folate[e] (μg/g)	Liver folate[e] (μg)	Plasma folate[e] (ng/ml)
Control[b]	—	14.7 ± 0.8	10.6 ± 0.6[f]	152 ± 5	44 ± 3[f]
Basal[a]	—	14.2 ± 0.5	4.9 ± 0.6[g]	70 ± 10	72 ± 4[g]
	B_{12}[c]	16.6 ± 0.4	3.9 ± 0.6[g]	64 ± 11	63 ± 4[g]
	Me[d]	16.4 ± 0.7	5.0 ± 0.8[g]	78 ± 9	50 ± 3[f]
	B_{12} + Me	15.6 ± 0.7	8.0 ± 0.7[f]	123 ± 8	45 ± 1[f]

[abcd] See corresponding footnotes for Table I.

[e] Values are mean ± SE for eight animals. Liver and plasma folates were measured using *L. casei*. Liver was pretreated with hog kidney conjugase. For each parameter, means bearing different letters are significantly different ($p<0.01$).

erage increment of 48 μg of folate/liver in 30 hr.

Expt. 2. The effects of intraperitoneal injections of vitamin B_{12} and methionine on net liver uptake of ^{3}H folic acid are shown in Table III. Separate injections of 40 mg of methionine or 2.5 μg of B_{12} did not alter ^{3}H folic acid uptake significantly, but when given together, uptake was restored to control values (+B_{12}, +Me diet) within 10 hr. Although 40 mg of methionine did not increase ^{3}H folate uptake in the absence of supplemental vitamin B_{12}, 225 mg of methionine was effective when given alone, and no further increase in folate uptake was obtained with additional vitamin B_{12} (Table III).

Discussion. In both experiments the animals fed the +B_{12}, +Me diet acted as controls to furnish normal values of ^{3}H folic acid uptake, liver folate, and plasma folate concentrations for comparison with other treatments. The ^{3}H folic acid uptake values of 17.2 and 11.4%/10 hr for such animals (Tables I and III) compare favorably with those obtained by Yoshino (8), and folate levels of 10.6 μg/g (conjugase treated) and 44 mg/ml for liver and plasma, respectively, are within the normal range (5, 9, 19).

After feeding the —B_{12}–methionine deficient, and this resulted in low liver ^{3}H folic acid uptake (Tables I and III), and, consequently, low liver folate concentrations (Table II). It is striking that these changes occurred despite an abundant supply of folic acid (5 mg/kg) in the diet, and elevated folate levels in plasma (Table II). Our observations of liver folate changes confirm those of Kutzbach *et al.* (4)

Liver uptake of ^{3}H folic acid as measured in the present experiments is the net result of the processes of folic acid entry into liver, its reduction and conversion to various tetrahydro forms (both monoglutamate and polyglutamate) and the reverse process of diff-

TABLE III. The Effect of Intraperitoneal Supplements of Vitamin B_{12} and Methionine on Uptake of Intravenously Administered ^{3}H Folic Acid by Rat Liver.

Diet fed 15 weeks	Supplement, ip, for 10 hr	^{3}H Folate uptake[f] (% in 10 hr)	Relative uptake (%)
Control[b]	—	11.4 ± 0.8[g]	100
Basal[a]	—	4.1 ± 0.3[h]	36
	B_{12}[c]	5.1 ± 0.4[h]	45
	Me (40 mg)[d]	5.5 ± 0.5[h]	48
	B_{12} + Me (40 mg)	13.1 ± 1.1[g]	115
	Me (225 mg)[e]	17.8 ± 1.8[i]	156
	B_{12} + Me (225 mg)	18.0 ± 0.7[i]	158

[a] —B_{12}, —Me diet: deficient in Vitamin B_{12}, only 0.2% methionine.

[b] +B_{12}, +Me diet: basal diet with 100 μg of B_{12}/kg and 0.7% methionine added.

[c] Intraperitoneal injection of 2.5 μg of vitamin B_{12}.

[d] Intraperitoneal injection of 40 mg of methionine.

[e] Intraperitoneal injection of 225 mg of methionine.

[f] Values are mean ± SE for eight animals. Means with different letters are significantly different ($p<0.01$).

usion of folates into the plasma. The experiments of Yoshino (8) indicate that after a single dose of ^3H folic acid the uptake of radioactivity by the liver is most rapid between 0–6 hr and continues to rise slowly for the next 18 hr. Thus, in the 10-hr period used in our experiments the entry and interconversion processes probably predominate over exit processes, especially since ^3H folic acid entering the liver is transformed and mixed with a relatively large pool of unlabeled folates. The observed reduction in ^3H folic acid uptake during vitamin B_{12}–methionine deficiency therefore reflects an impairment in the rate of entry and/or interconversion of liver folates.

Jeejeebhoy *et al.* (11) found that vitamin B_{12} deficiency caused a comparatively greater depletion of polyglutamate rather than monoglutamate folates in human red cells, and Kutzbach *et al.* (4) noted a similar effect in rat liver. Both groups of authors were led to speculate that vitamin B_{12} is important for the maintenance of adequate tissue concentrations of polyglutamate forms of folate. Similarly, our results could be explained by postulating a role for vitamin B_{12} and/or methionine in promoting the conversion of folate monoglutamates to polyglutamates. Because of their larger molecular size, polyglutamates may be more easily retained by the liver. The reduced rate of polyglutamate production during vitamin B_{12} and/or methionine deficiency would then result in low liver folate levels and reduced net uptake of ^3H folic acid. The alternative explanation of an effect of vitamin B_{12} and/or methionine on the folate transport mechanism in the cell membrane seems less likely, but cannot be ignored.

It is interesting that although 40 mg of methionine given intraperitoneally did not stimulate the ^3H folic acid uptake of deficient animals unless accompanied by 2.5 μg of vitamin B_{12}; 225 mg of methionine was completely effective when given alone, and additional vitamin B_{12} had no further effect. This suggests that methionine is the primary factor affecting the uptake and storage of folates, and vitamin B_{12} acts indirectly via its role in *de novo* synthesis of methionine when the exogenous supply is inadequate.

It may be argued that changes in the uptake of intravenously injected ^3H folic acid do not represent physiological changes because the predominant form of folate in plasma is 5-methyltetrahydrofolic acid, rather than folic acid itself. However, this is not a serious drawback because changes in total liver folate levels (Table II), which represent changes in physiological forms of folate, paralleled changes in ^3H folic acid uptake (Tables I and II). Furthermore, it has not yet been established that 5-methyltetrahydrofolic acid is the form of folate that is absorbed from the alimentary canal in rats. Dietary folic acid may be absorbed unchanged into the portal blood and undergo conversion to other forms such as 5-methyltetrahydrofolic acid by the liver. Whitehead and Cooper (12) claim that this process occurs in humans.

The action of vitamin B_{12} and methionine supplements in reducing the formiminoglutamic acid (FIGLU) and aminoimidazolecarboxamide (AIC) excretion of vitamin B_{12} deficient animals has been established (13–16). Explanations of the mode of action of these supplements to date have not considered changes in the size of the liver folate pool. The results in Table III show that methionine supplements can cause a very significant increase in ^3H folic acid uptake by the liver. Also, in the presence of adequate methionine, vitamn B_{12} supplements can produce an increase of 48 μg of folate/liver in 30 hr (Table II). This represents a 68% expansion in the liver folate pool. Although the form(s) of incoming folate is not known, it is likely that directly, or indirectly, additional tetrahydrofolic acid would be made available within the liver for FIGLU and AIC catabolism, thereby decreasing the amounts of these substances excreted in the urine.

Summary. The liver uptake of intravenously administered ^3H folic acid in rats fed a vitamin B_{12}–methionine diet was only 55% of that found in normal rats. Supplementation of the deficient diet for 30 hr with 100 μg of B_{12}/kg did not alter folate uptake significantly, but if vitamin B_{12} was fed together with 0.7% methionine, the rate of uptake was rapidly increased to control values. When uptake was stimulated in this way it was

observed that plasma folate levels decreased from 72 to 45 ng/ml and liver folate concentrations increased from 5 to 8 μg/g—an increment of 48 μg of folate/liver in 30 hr. Intraperitoneal injection of 2.5 μg of B_{12} and 40 mg of methionine together in deficient animals, demonstrated that the depressed rate of folate uptake could be restored to control values even within 10 hr. Injection of 225 mg of methionine stimulated ^3H folic acid uptake in the absence of any additional vitamin B_{12}. It is suggested that vitamin B_{12} and/or methionine can influence folate metabolism in rat liver by altering the size of the folate pool.

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