Influence of Vitamin B_{12} and Methionine on Levels of Folic Acid Compounds and Folate Enzymes in Rat Liver.* (31857)

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The close relation between the functions of vitamins B_{12} and folic acid is best documented by biochemical deficiency symptoms which are common to both, such as the increased urinary excretion of formimino-glutamic acid (FIGlu),† aminoimidazole-carboxamide (AIC) and formate(1-3). In both deficiencies, dietary methionine diminishes or even abolishes these symptoms(4-6). The action of methionine may be explained by its effect on the tissue distribution of folic acid coenzymes. Noronha and Silverman(7) showed that supplementation of rats with 1% D,L-methionine for 24 hours decreased the level of 5-methyl-tetrahydrofolate (5-CH_3-H_4folate) and increased the levels of other forms active in the metabolism of the excreted compounds. The exact site of methionine action has, however, not yet been identified.

The levels of 5-CH_3-H_4folate appear to be controlled by 2 enzymes, methylenetetrahydrofolate reductase (E.C.1.1.1.68) (I) and methyl-tetrahydrofolate:L-homocysteine S-methyl transferase (transmethylase) (II). The reductase is most probably irreversible under physiological conditions(8).

(I) 5,10-CH_2-H_4folate + $FADH_2$ → 5-CH_3-H_4folate + FAD

(II) 5-CH_3-H_4folate + L-homocysteine $\xrightarrow[SAM]{B_{12}\ \text{enzyme}}$ H_4folate + L-methionine

It has been proposed that methionine enhances utilization of 5-CH_3-H_4folate by providing the activator SAM and/or the acceptor homocysteine for reaction II(7,9). Yet it is difficult to understand how this effect alone would overcome the limitation of the low enzyme II level caused by B_{12} deficiency (10). Methionine, however, appears to repress enzyme I in *E. coli*(8,11). There results suggested a possible regulatory influence of methionine at this site in rats.

This report investigates in rats the effects of B_{12} and methionine on the levels of enzymes I and II, as well as on the level of glutamate formimino-transferase (E.C.2.1.2.5) (III) (FIGlu + H_4folate ⇄ 5-HCNH-H_4folate + L-Glu). Enzyme II was studied to compare the results with those of Dickerman with chicks(10). Enzyme III was measured because a direct influence on vit B_{12} on this enzyme has been proposed(12). Microbiological analyses of folates in the livers of the same animals were performed with recently published improved methods which avoid the hydrolysis of polyglutamates during preparation of the extracts(13). The data reported here show the role of B_{12} and methionine in controlling transmethylase activity and their pronounced effect on folate levels in liver. No effects on reductase (I) and formimino-transferase (III) were observed.

Materials and methods. Diets: The basal diet had the following composition (g/kg): soy assay protein,‡ 200 g; corn oil with vitamins A, D, E, 40 g; salt mix(14) 35 g; vitamin premix in Cerelose,§ 10 g; choline chloride, 1 g; Cerelose, 714 g. The vitamins were supplied in the following concentrations (per kg): vit A, 15,000 I.U.; vit D (Viosterol), 200 units; tocopherol, 50 mg; biotin, 0.2 mg; thiamine, 15 mg; riboflavin, 15 mg; niacin, 50 mg; pyridoxine, 15 mg; Ca-pantothenate, 50 mg; folic acid, 5 mg; menadione, 10 mg. Vit B_{12} was added where indicated as 100 mg of a 0.1% trituration in mannitol corresponding to 100 μg/kg. D,L-methionine was added as 15 g/kg where indicated. Some animals received higher methionine supple-

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† *Abbreviations:* AIC, 4-Amino-5-imidazole carboxamide; FAD, Flavin-adenine-dinucleotide; FIGlu, N-formimino-L-glutamic acid; Glu, L-glutamic acid; PteGlu, Pteroylglutamic acid; SAM, S-adenosyl-L-methionine. (Abbreviations of folic acid derivatives are according to the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature, as published in Biochim. Biophys. Acta 107, 11 (1965).)

‡ Obtained from General Biochemicals.

§ Cerelose—trade name for glucose monohydrate.

ments for the last 2-3 weeks before sacrifice. A slight weight loss was observed on 3% and 4% methionine diets.

In the "labile methyl-free" diet, choline was omitted and vit B_{12} added, and the protein replaced by an amino acid mixture complete in essential amino acids except that homocysteine replaced methionine. The amino acid mixture(15) furnished per kg of diet: L-arginine × HCl, 8 g; L-cystine, 3 g; L-glutamic acid, 60 g; glycine, 6 g; L-histidine × HCl, 7 g; D,L-isoleucine, 14 g; L-leucine, 10 g; L-lysine × HCl, 14 g; D,L-homocystine, 12 g; D,L-phenylalanine, 12 g; D,L-threonine, 12 g; D,L-tryptophane, 2 g; D,L-valine, 18 g; L-tyrosine, 4 g; D,L-serine, 5 g; sodium bicarbonate, 12.4 g.

Animals. Two-weeks-pregnant Sprague-Dawley rats, obtained from a commercial breeder, were fed the basal diet supplemented with an additional 50 g soy assay protein and 7 g D,L-methionine per kg diet until weaning at 3 weeks. Each litter was reduced to 8 animals at 1 day after birth. The animals were assigned to the experimental diets, and for the first 5 weeks were kept 2 in a cage; subsequently, individually in wire-bottom cages. Food and water were given *ad libitum*. The animals were sacrificed by decapitation, 1 from each group at 11 weeks, and the remainder in the 14th and 15th weeks. The livers were removed immediately and cooled in ice. Five g of each liver was taken for folic acid analysis and an extract prepared by immediate homogenization with ascorbate solution and subsequent heating to 95°C for 10 minutes according to the method given by Bird *et al*(13). The remainder was used for enzyme assay and homogenized with 3 volumes of cold 0.05 M phosphate buffer pH 7.2 in a Potter-Elvehjem homogenizer and centrifuged at 30,000 × *g* for 30 minutes. The supernatants were frozen at —15°C until assayed. (Not all samples were assayed but all 3 enzymes were always assayed on one sample.)

Enzyme assays. Immediately before analysis, part of each sample was dialyzed 2 hours against 2 liters of 0.05 M phosphate buffer pH 7.2 at 4°C. The transmethylase assay was adopted in principle from Dickerman *et al*(10). The incubation mixture contained 60 mμmoles $\pm^{14}CH_3$-H_4PteGlu (543 dpm/mμmole), 500 mμmoles D,L-homocysteine (dissolved fresh daily), 100 mμmoles SAM, 1 μmole ascorbate, 10 μmoles K-phosphate buffer pH 7.2, 100 mμmoles catalytically reduced FAD and an amount of enzyme, which produced maximally 15 mμmoles methionine, in a total volume of 0.3 ml. Incubation procedure was according to Katzen and Buchanan(8) in which the reaction vessels, closed with a rubber cap, were filled with pure hydrogen by repeated evacuation and filling through a 3-way stopcock and the reduced FAD injected with a syringe. After 2 hours incubation at 35°C, the content of the vessels was passed over a 0.5 × 2 cm column of Dowex 1-CL (100-200 mesh), which retains the $^{14}CH_3$-H_4PteGlu and lets the methionine pass through, and the columns washed with water to give a total eluant volume of 3.0 ml. One ml of this eluant was dried on a planchet under an infrared lamp and counted in a Nuclear-Chicago gas-flow counter type 161A. A blank value without enzyme was subtracted. The assay response curve was linear with respect to time and to the amount of protein in the range used.

Methylene-tetrahydrofolate reductase was assayed in the reverse direction with menadione as the artificial electron acceptor(16). ^{14}C-formaldehyde formed from $^{14}CH_3$-H_4Pte Glu was determined as the dimedone adduct which could be extracted with toluene(8). A similar method of ^{14}C-formaldehyde determination has been published by Taylor and Weissbach(17). The incubation mixture contained(final volume, 0.55 ml) 320 mμmoles of $\pm^{14}CH_3$-H_4PteGlu (110 dpm/mμmole) 50 mμmoles FAD, 2 μmoles menadione (not completely dissolved), 5 μmoles ascorbate, 100 μmoles phosphate buffer pH 6.3, 1 μmole ethylenediaminetetraacetic acid and enzyme, which produced a maximum of 30 mμmoles formaldehyde. After incubation for 1 hour at 35°C in air, 0.3 ml of a solution of 3 mg dimedone per ml in 1 M K-acetate buffer pH 4.5 were added, the mixture heated for 5 minutes at 95° and cooled in ice. The formaldehyde-dimedone adduct was extracted

into 3 ml of toluene by vigorous shaking with a Vortex mixer for 15 seconds. The phases were then separated by centrifugation and 1 ml of the toluene phase transferred to a counting vial with 5 ml of scintillator solution in toluene, and the samples counted in a Packard Tricarb Scintillation Counter Model 314-DC. A blank value without enzyme was subtracted. The test was nearly linear with time but not with protein. It was found that the protein interfered with the recovery of the formaldehyde as the dimedone adduct. A recovery curve was made using ^{14}C-formaldehyde + H_4PteGlu instead of the substrate and used for correction of the enzyme activities. Formimino-transferase was determined spectrophotometrically according to Tabor and Wyngarden(18). The incubation contained 2 μmoles $\pm H_4$PteGlu, 5 μmoles FIGlu, 200 μmoles mercaptoethanol, 100 μmoles phosphate buffer pH 7.2 and enzyme, which produced maximally 100 mμmoles product in a total of 1.0 ml. Incubation was carried out for 15 minutes at 35°C; 0.3 ml of 10% perchloric acid were added to stop the reaction and the mixture heated for 55 seconds in a boiling water bath, then rapidly cooled, centrifuged and the optical density measured at 355 mμ. Results were calculated using a molecular extinction coefficient of $\epsilon = 25 \cdot 10^3$ 1 Mol^{-1} cm^{-1} for 5,10-methenyl-H_4PteGlu. A blank without FIGlu was subtracted.

Protein was determined by the Biuret method as described by Beisenherz *et al*(19).

FIGlu excretion was determined on 24-hour urine collections made in flasks containing 0.5 ml of 1 M sulfuric acid to stabilize the FIGlu. The assay was carried out according to Tabor and Wyngarden(20).

Folate analyses. Microbiological folate analyses using *Lactobacillus casei* ATCC 7469, *Pediococcus cerevisiae* ATCC 8081 and *Streptococcus faecalis* ATCC 8043, were performed by the "aseptic addition" technique according to Bakerman(21), using a final volume of 5 ml, however. Difco folic acid casei medium and -CF assay medium were used. In all assays, d,l-Ca-leucovorin was the standard and calculated as the l-form. The preparation of the hog kidney conjugase and the method of digestion of the extracts were according to Bird *et al*(13).

Materials. Unlabeled and labeled $\pm$5-CH_3-H_4PteGlu were prepared by reduction of a mixture of H_4PteGlu and formaldehyde with sodium borohydride(22) and purification on TEAE cellulose with ammonium bicarbonate as eluant. The products were obtained as barium salts.‖ ^{14}C-formaldehyde, SAM iodide, FAD and FIGlu were obtained from Calbiochem. D,L-homocysteine and the H_4PteGlu used in the FIGlu assays were from Nutritional Biochemicals Corp. H_4PteGlu for preparation of 5-CH_3-H_4PteGlu was made by catalytic hydrogenation of folic acid in neutral aqueous solution(23) and used without isolation. d,l-leucovorin was a gift of the Lederle Laboratories, American Cyanamid Corp., Pearl River, N. Y.

Results and discussion. 1. *Enzyme levels.* The levels of methylenetetrahydrofolate reductase and formimino-transferase were similar on all of the diets (Table I). This indicates that the synthesis and function of these enzymes is not influenced by vit B_{12} or methionine. Similar observations on the reductase have been reported by Dickerman (24) on chicks. Dialysis had no effect on the activity of the transferase but caused a 1.5-2-fold increase in reductase activity. Addition of a lyophilized concentrate of dialysate did not, however, inhibit the enzyme *in vitro*.

The levels of transmethylase were significantly lower in the vitamin B_{12} deficient groups, in agreement with the results with chicks(10). Addition of vit B_{12} to the *in vitro* test system had no effect. Methionine supplementation decreased enzyme activity. The effect with 4% methionine was no greater than with 1.5%. Dialysis did not affect transmethylase activity.

It had been shown that rats on a diet containing neither methionine nor choline but a sufficient supply of homocysteine, vit B_{12} and folic acid grow normally(25) and synthesize 4 times more choline-methyl groups from one-carbon precursors than when methi-

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TABLE I. Enzyme Levels in Dialyzed Rat Liver Homogenate Supernatant.

Group	No. rats	Diet	Supplements B_{12}	Supplements Met	Avg body wt at 10 wk	Enzyme activities (μmoles min^{-1} mg^{-1} ± S.D. ($\times 10^3$)) Reductase	FI-transferase	Trans-methylase
1	4	basal	—	—	303 ± 39	.29 ± .06	132 ± 10	.06 ± .02
2	4	"	—	1.5%	386 ± 48	.31 ± .09	146 ± 10	.05 ± .01
3	8	"	+	—	352 ± 24	.30 ± .11	172 ± 53	.27 ± .12
4	9	"	+	1.5–4%	367 ± 26	.23 ± .04	139 ± 50	.13 ± .05
5	5	labile methyl-free	+	—	239 ± 17	.35 ± .09	185 ± 66	.33 ± .16

onine or choline is supplied(26). The rats in group 5 were therefore transferred at 5 weeks from the basal diet (without B_{12} and without methionine) to the methionine- and choline-free amino acid diet supplemented with vit B_{12} to determine whether this would cause a further increase in transmethylase activity. Weight gain of the animals stopped for approximately 10 days and then resumed at about the same rate as before. The transmethylase activity in this group after 6 to 9 weeks on the labile methyl-free diet, however, was no greater than that in group 3 fed the basal diet providing 0.2-0.3% methionine.

The observed effects of the dietary methionine supply on the enzyme responsible for its formation are small and therefore would not represent an efficient regulation of methyl group biosynthesis. Strong control of methyl group biosynthesis at this site might be disadvantageous to the cell in view of the metabolic importance of the release of free H_4folate from the storage form of 5-CH_3-H_4folate(27).

2. *Folate level studies.* Animals on the vitamin B_{12}-deficient basal diet had significantly lower levels of all forms of folate in comparison with the values from the supplemented diets (Table II). By comparison with data from rats on a stock diet(13), the latter can be assumed as normal. The effect of methionine on *Pediococcus cerevisiae* activity representing CHO-H_4folate and H_4folate was much less than that found by Noronha and Silverman(7) after 24-hour supplementation with 1% methionine. The latter report is the only one correlating the composition of liver folates with methionine and vit B_{12} intake in a purified diet. Our investigation differs from theirs in that the duration of the methionine supplementation in these investigations was longer, and in the fact that they did not differentiate between mono- and higher glutamates of folates since the latter are split down during freezing and thawing of the livers in their assay procedure(13).

Our most striking observation, however, was the very low total folate content of groups 1 (basal diet) and 5 (labile methyl-free, + B_{12}) in spite of supplementation with 5 mg folate/kg diet. In these groups, not only the monoglutamates but mainly the higher conjugates were reduced, as is shown by the difference in *L. casei* activity before and after conjugase treatment. Methionine alone is almost as effective as vit B_{12} in maintaining normal levels. However, despite the low level of *P. cerevisiae* activity in groups 1 and 5, FIGlu excretion of 3-60 μmoles per day oc-

TABLE II. Folate Levels in Rat Liver Extracts.

Group	No. rats	Diet	Supplements B_{12}	Supplements Met	μg l-Leucovorin activity/g liver ± S.D. Conjugase-untreated *L. casei*	*P. cer.*	*S. faec.*	Conjugase-treated *L. casei*	*P. cer.*
1	6	basal	—	—	3 0 ± 1.1	1.3 ± .9	.9 ± .7	3.8 ± .8	2.3 ± .9
2	6	"	—	+1.5%	5.9 ± 1.9	2.8 ± .7	3.0 ± .6	10.7 ± 4.7	6.2 ± 2.5
3	6	"	+	—	7.3 ± 3.3	2.0 ± 1.2	4.0 ± 1.6	12.1 ± 3.1	5.1 ± 2.3
4	8	"	+	1.5–4%	7.8 ± 1.3	2.5 ± 1.0	4.7 ± 1.2	15.7 ± 5.4	6.6 ± 2.6
5	5	labile methyl-free	+	—	2.6 ± .9	.9 ± .5	.9 ± .4	6.6 ± 4.2	2.6 ± 1.7

curred only in the B_{12}-deficient group 1; animals in all other groups excreted below 1 μmole per day. Possibly the presence of B_{12} in group 5 allowed a faster turnover of the methyl-folate and thus more effective utilization. Somewhat comparable results in the composition of whole blood folates have recently been reported by Jeejeebhoy *et al*(28). The ratio of conjugated to unconjugated folates dropped from 10/1 in normals to 1.3/1 in patients with pernicious anemia.

Little is known of the biosynthesis of the conjugates in higher organisms, the only *in vitro* studies being done on *E. coli*(29). In that case it was shown that glutamic acid was added stepwise to folate only in the tetrahydro-form; 5-formyl- or 5-methyl-H_4 folate did not react. If it could be shown that 5-methyl-H_4PteGlu is inactive as a substrate for the polyglutamate-forming system in rats, the effect of B_{12} and methionine would easily be explained by their influence on tissue levels of free H_4folate in exactly the same way as in the case of excretion of FIGlu, AIC and formate.

Summary. 1. The level of methionine-forming enzyme (transmethylase) activity in rat liver is dependent on vitamin B_{12} and is decreased by dietary methionine in much the same way as reported for chickens(10). The enzyme levels of methylene-tetrahydrofolate reductase and glutamate formiminotransferase are not influenced by either vitamin B_{12} of methionine. 2. Rats kept on a vitamin B_{12}-deficient diet low in methionine for 11 weeks show a marked drop in liver folate levels, especially higher conjugates, compared to animals supplemented with either vitamin B_{12} or methionine, despite high folic acid content in the diet.

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