The Oxidation of 5-Methyl-^{14}C-tetrahydrofolate and Histidine-2-^{14}C to $^{14}CO_2$ in Vitamin B_{12}-Deficient Rats[1] (34759)

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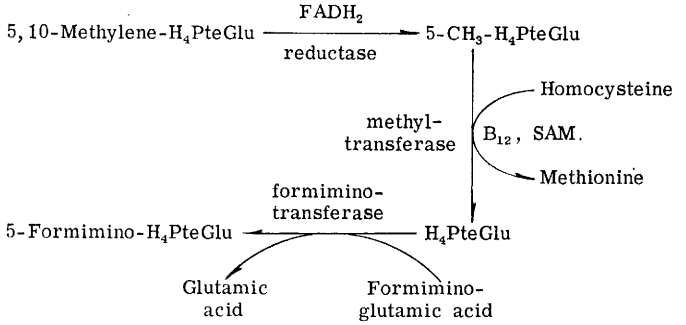
Evidence for a metabolic interrelationship between vitamin B_{12} and folic acid is abundant. The macrocytic, megaloblastic anemia seen in humans is caused by a deficiency of either vitamin (1), as is the elevated urinary excretion of formiminoglutamic acid (FIGlu)[4] in humans, rats and chicks (2–6). The essential amino acid methionine is involved in the interrelationship since its presence in adequate or excess amounts can restore to normal the high urinary FIGlu excretion in rats (3, 7) and humans (8). The only known metabolic pathway common to folic acid, vitamin B_{12}, and methionine includes the 5-methyltetrahydrofolate: L-homocysteine *S*-methyltransferase reaction shown in the following scheme:



5-Methyl-H_4PteGlu is formed from 5,10-methylene-H_4PteGlu by a reductase enzyme requiring $FADH_2$, and the 5-methyl-H_4PteGlu donates its methyl group to homocysteine to form methionine and H_4PteGlu. This methyltransferase reaction requires vitamin B_{12} and *S*-adenosylmethionine as cofactors in mammalian systems. The released H_4PteGlu can be utilized by the formiminotransferase enzyme to accept the formimino group from FIGlu with the formation of 5-formimino-H_4PteGlu and glutamic acid.

Several investigators have discussed the possibility that a metabolic deficiency of folic acid exists in vitamin B_{12} deficiency as a consequence of impairment in the vitamin B_{12}-dependent methyltransferase reaction. *In vitro* evidence shows that methylenetetrahydrofolate reductase is essentially irreversible (9, 10), and it is therefore contended that accumulation or "trapping" of metabolically active folates such as 5-CH_3-H_4PteGlu occurs (11–14). If these current concepts are correct, changes in the rate of catabolism of compounds such as histidine, that require H_4PteGlu for one-carbon transfer, will follow changes in the rate of the methyltransferase reaction.

In order to examine this prediction we have compared the $^{14}CO_2$ production from histidine (imidazole-2-^{14}C) with that from 5-$^{14}CH_3$-

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[4] Abbreviations: FIGlu, formiminoglutamic acid; H_4PteGlu, tetrahydrofolic acid; 5-CH_3-H_4PteGlu, 5-methyltetrahydrofolic acid; SAM, *S*-adenosylmethionine; $FADH_2$, flavin-adenine dinucleotide (reduced).

H_4PteGlu in rats fed diets differing in vitamin B_{12} and methionine content, and thus likely to cause changes in the rate of the methyltransferase reaction.

Materials and Methods. Diets. The basal diet had the following composition (g/kg): soy assay protein,[5] 200; glucose monohydrate, 714; corn oil with vitamins A, D and E, 40; salt mix (15), 35; vitamin premix 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; biotin, 0.2 mg; thiamine·HCl, 15 mg; riboflavin, 15 mg; pyridoxine·HCl, 15 mg; Ca-pantothenate, 50 mg; niacin·HCl, 50 mg; menadione, 10 mg; folic acid, 5 mg. When the basal diet was supplemented with vitamin B_{12} and/or D,L-methionine, the levels were 100 μg/kg and 3 g/kg, respectively.

Animals. Male, weanling rats of the Sprague-Dawley strain[6] were housed singly in stainless steel screen-bottom metabolism cages in a constant temperature and light-controlled room. Four animals were assigned to each of the 4 diet groups described in Table I and fed the diets and water *ad libitum* for 8 weeks before commencing the metabolic tests.

Methylmalonic acid determination. A 24-hr urine collection was made from each rat after 8 weeks on the diets and methylmalonic acid analysis was performed as evidence of vitamin B_{12} deficiency. The method of Giorgio and Plaut (16) was used with an adaptation for automated analysis. One- to 2-ml urine samples were passed through 3 × 1.3-cm columns of Bio-Rex 5[7] anion exchange resin, 100–200 mesh. The methylmalonic acid was eluted with 25 ml of 0.2 *N* HCl and assayed directly with an autoanalyzer using diazotized *p*-nitroaniline reagent for the color reaction.

$^{14}CO_2$ studies. The radioactive compounds administered were L-histidine (imidazole-2-^{14}C), 57.8 mCi/mmole and 5-$^{14}CH_3$-H_4PteGlu (barium salt), 50.8 mCi/mmole.[8] Continuous tracings of pulmonary $^{14}CO_2$ were recorded using a technique developed by Tolbert *et al.* (17). Each animal was placed in a glass metabolism chamber after injection of the radioactive compound and the expired air was measured for radioactivity using a flow system ionization chamber with a vibrating reed electronmeter[9] and a strip chart recorder.[10] The total CO_2 was collected for a 60-min period using an ethanolamine–ethylene glycol monomethyl ether trapping solution and then counted for radioactivity with a scintillation counter (18).

On day 1 of the CO_2 collection study, each rat was injected in the femoral vein with 2 μCi (35 mμmoles) of 5-$^{14}CH_3$-H_4PteGlu, in 0.2 ml. On day 2, each animal was injected intraperitoneally with 2 μCi (39 mμmoles) of the same concentration of histidine-2-^{14}C. Both solutions were in physiological saline.

Results. The lack of dietary vitamin B_{12} affected both growth and methylmalonic acid excretion (Table I).

The mean body weight of animals in the $+B_{12}$ +Met diet group was greater than that of animals in the 3 groups that lacked adequate vitamin B_{12} and/or methionine. Daily urinary methylmalonic acid excretion doubled in animals fed diets deficient in vitamin B_{12}, regardless of the methionine status.

The $^{14}CO_2$ excretion tracings (Fig. 1) revealed that the rate of $^{14}CO_2$ production from histidine-2-^{14}C was greatly reduced in animals fed the $-B_{12}$ –Met diet relative to those supplemented with vitamin B_{12} and methionine. $^{14}CO_2$ excretion tracings for 5-$^{14}CH_3$-H_4PteGlu were similar except that the T_{max} was delayed in the doubly deficient animals (Fig. 1).

A summary of the data obtained from individual animals is shown in Tables II and III. After injection of histidine-2-^{14}C, the maximum rate of $^{14}CO_2$ production and percent-

[5] Obtained from General Biochemicals, Chagrin Falls, Ohio; Contains 1.0% methionine.

[6] Simonson Laboratories, Gilroy, Calif.

[7] Obtained from Bio-Rad Laboratories, Richmond, Calif.

[8] Nuclear-Chicago Corp., Des Plaines, Ill.

[9] Cary Model 31, Applied Physics Corp. Monrovia, Calif.

[10] Electronik 15, Honeywell, Inc., Philadelphia, Pa.

TABLE I. The Effect of Vitamin B_{12} and Methionine on Growth and Methylmalonic Acid Excretion in Rats.

Diet group	No. of rats	Basal diet supplements: Vit B_{12} (100 μg/kg)	Basal diet supplements: D,L-Met (3 g/kg)	% Total methionine	Body wt (g)	24-hr urinary methylmalonic acid (μmoles/100 g of body wt)
$-B_{12}$ —Met	4	—	—	0.2	316 ± 24[a]	21.4[b]
$+B_{12}$ —Met	4	+	—	0.2	308 ± 27	8.4[c]
$-B_{12}$ +Met	4	—	+	0.5	319 ± 31	16.5[b]
$+B_{12}$ +Met	4	+	+	0.5	348 ± 30	7.2[c]

[a] ± Standard error of the mean $= \{[\Sigma X^2-(\Sigma x)^2/n]/[n(n-1)]\}^{1/2}$.

[bc] Means with different superscripts are significantly different ($p < .02$).

age of dose expired in 60 min by animals in the $-B_{12}$ —Met group was 1/10 of corresponding values for groups supplemented with vitamin B_{12} and/or methionine. The T_{max} was not significantly changed by any of the dietary treatments.

In contrast, when 5-$^{14}CH_3$-H_4PteGlu was injected, the maximum rate of $^{14}CO_2$ production and percentage of dose expired in 60 min was unaffected by dietary treatment, but the T_{max} was delayed in animals lacking dietary vitamin B_{12}. Methionine supplementation only slightly affected the T_{max}.

Histidine-2-^{14}C and 5-$^{14}CH_3$-H_4PteGlu were injected in approximately equal amounts, yet the maximum rate of $^{14}CO_2$ production and the percentage of dose expired in 60 min was 3 times higher after injection of the former than after injection of the latter, in all groups except the $-B_{12}$ —Met group where histidine catabolism was severely impaired.

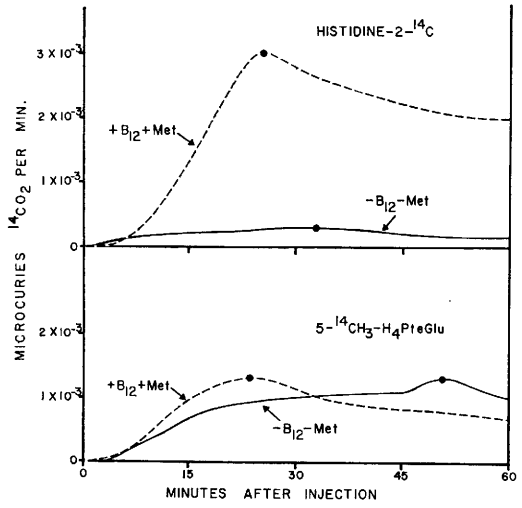

FIG. 1. Pulmonary $^{14}CO_2$ production from L-histidine-2-^{14}C or 5-$^{14}CH_3$-H_4PteGlu in rats fed a diet either deficient or supplemented with vitamin B_{12} and D,L-methionine. The T_{max} is indicated by a closed circle for each curve.

Discussion. Elevated methylmalonic acid excretion has gained acceptance as a specific diagnostic of vitamin B_{12} deficiency (19–22).

TABLE II. Pulmonary $^{14}CO_2$ Excretion After Intraperitoneal Injection of Histidine-2-^{14}C.

Diet group	T_{max} (min)	Maximum rate (μCi/min)	% Dose expired in 60 min
$-B_{12}$ —Met	26.4	0.32×10^{-3}[a]	0.79[c]
$+B_{12}$ —Met	29.2	3.37×10^{-3}[b]	6.31[d]
$-B_{12}$ +Met	39.3	3.91×10^{-3}[b]	7.15[d]
$+B_{12}$ +Met	29.4	3.20×10^{-3}[b]	6.23[d]

[abcd] Means for each column with different superscripts are significantly different ($p < .01$).

TABLE III. Pulmonary $^{14}CO_2$ Excretion After Intravenous Injection of $^{14}CH_3$-tetrahydrofolate.

Diet group	T_{max} (min)	Maximum rate (μCi/min)	% Dose expired in 60 min
$-B_{12}$ —Met	49.6[a]	1.08×10^{-3}[c]	2.12[d]
$+B_{12}$ —Met	28.2[b]	0.89×10^{-3}	2.25
$-B_{12}$ +Met	41.1[a]	0.97×10^{-3}	1.90
$+B_{12}$ +Met	30.9[b]	1.01×10^{-3}	1.93

[ab] Means with different letters are significantly different ($p < .01$).

[cd] No significant differences in mean values.

Using this criterion, animals in the $-B_{12}$ $-$Met and $-B_{12}$ $+$Met diet groups were definitely deficient in vitamin B_{12}. The reduction in histidine-2-^{14}C oxidation observed in the former group (Fig. 1 and Table II), despite 5 mg of folic acid/kg of diet, is indicative of secondary folic acid deficiency. We have also observed a large reduction in total folate levels in the livers of animals fed a similar diet (*23*).

The rate of oxidation of a ^{14}C-labelled compound such as histidine-2-^{14}C or 5-^{14}CH$_3$-H$_4$PteGlu to $^{14}CO_2$ in the intact animal is a function of the rate of several biochemical steps. Not only is it dependent on the activity of the rate-limiting enzyme involved in the catabolism of the organic compound to CO_2, but it is also dependent on the pool sizes of the injected compound and its products, the rate of transport of the compound into the tissues where oxidation occurs and on the rate of CO_2 production from bicarbonate. We can represent these first order, consecutive reactions as follows:

$$A \xrightarrow{k_1} H^{14}CO_3^- \xrightarrow{k_2} {}^{14}CO_2. \quad (1)$$

A is the isotopic organic compound injected, k_1 is the rate constant for the sum of the metabolic processes involved in the uptake and oxidation of A, and k_2 is the rate constant for the production of $^{14}CO_2$ from ^{14}C bicarbonate. In our experiment, we have assumed that k_2 is constant and greater than k_1 in all groups. Therefore, changes in the rate-limiting step alters k_1.

T_{max}, which is independent of the specific activity of A, HCO_3^-, and CO_2, is related to the rate constants by the following equation:

$$T_{max} = \frac{\ln (k_2/k_1)}{k_2 - k_1}. \quad (2)$$

If $k_1 < k_2$ as we have assumed, then T_{max} is inversely related to k_1.

A delay in T_{max} has been reported by Fish *et al.* (*24*) for histidine-2-^{14}C oxidation in folic acid-deficient humans. Our results in vitamin B_{12}-deficient rats show no differences in T_{max} for histidine-2-^{14}C oxidation, with or without added dietary methionine, although the T_{max} for the $-B_{12}$ $-$Met group could not be accurately determined since the $^{14}CO_2$ tracings are nearly flat (Fig. 1).

The maximal rate of $^{14}CO_2$ production from histidine-2-^{14}C and the percentage of dose excreted in 60 min are dependent on the pool size of histidine and its metabolites such as FIGlu and also on the rate of urinary excretion of FIGlu. These may be expected to be increased in the $-B_{12}$ $-$Met group due to a block in the formiminotransferase reaction. Our data give evidence that one or both of these are increased in the $-B_{12}$ $-$Met group since the maximal rate of $^{14}CO_2$ production and the percentage of the dose excreted is reduced to 10% of the values in the groups supplemented with methionine and/or vitamin B_{12} (Table II). Brown *et al.* (*7*) have reported that the imidazole-2-^{14}C of histidine appears in urinary FIGlu in vitamin B_{12} deficiency in the same amount as in the pulmonary $^{14}CO_2$ in rats given methionine.

An interference with absorption or cellular transport of histidine by methionine has been proposed by Stahelin *et al.* to explain an observed reduction in histidine-2-^{14}C catabolism. Such an interference is unlikely in our diets because the added methionine brought the total content to only 0.5%, an amount just satisfying the requirement for the rat.

The T_{max} for the oxidation of 5-^{14}CH$_3$-H$_4$PteGlu to $^{14}CO_2$ was increased by vitamin B_{12} deficiency (Table III). From Eq. (*2*), the rate constant k_1 must have decreased. Such a decrease was probably due to an impairment in the methyltransferase reaction. *In vitro* evidence shows a reduction in the activity of this enzyme in vitamin B_{12}-deficient rats (*26*).

In contrast with the results obtained for histidine-2-^{14}C (Table II), the maximal rate of $^{14}CO_2$ production from 5-^{14}CH$_3$-H$_4$PteGlu and the percentage dose excreted in 60 min was not significantly altered by any dietary treatment. This contradicts what would be expected in vitamin B_{12} deficiency if current concepts are correct. According to the "methyl trap" hypothesis the 5-CH$_3$-H$_4$PteGlu pool size should be increased in vitamin B_{12} deficiency resulting in a decrease in specific activity of injected 5-^{14}CH$_3$-

H_4PteGlu, and consequently in the amount and rate of $^{14}CO_2$ produced. Since the labeled methyl group is transferred to homocysteine before oxidation, it would be expected that methionine supplementation would also affect the rate of production of $^{14}CO_2$ from 5-$^{14}CH_3$-H_4PteGlu. No such effect was observed (Table III). Our results for 5-$^{14}CH_3$-H_4PteGlu oxidation are not easily explained by present hypotheses unless a "tight" regulatory mechanism of 5-CH_3-H_4PteGlu metabolism is assumed.

Significantly, the methyl group of 5-$^{14}CH_3$-H_4PteGlu was oxidized at an equal rate in vitamin B_{12} deficiency with, or without, added methionine, while the imidazole-2-^{14}C of histidine was not. It is difficult to agree that there is a "methyl trap" in the simple terms of an accumulation of 5-CH_3-H_4PteGlu in tissues and a consequent reduction of H_4PteGlu for acceptance of the formimino group. The explanation must be more complicated and perhaps be related to the function of the other forms of folate, the tri- and polyglutamates, or a transport phenomenon which is dependent on vitamin B_{12} and/or methionine.

Summary. When rats were fed a diet deficient in vitamin B_{12} and limiting in methionine, the $^{14}CO_2$ production from L-histidine-2-^{14}C was reduced in comparison with rats fed diets supplementd with vitamin B_{12} and/or methionine. In contrast, the production of $^{14}CO_2$ from 5-$^{14}CH_3$-tetrahydrofolic acid was not altered by vitamin B_{12} deficiency or the level of dietary methionine. The T_{max} for the oxidation of 5-$^{14}CH_3$-tetrahydrofolic acid to $^{14}CO_2$ was delayed in vitamin B_{12} deficiency in comparison with normals, indicating that either an enzymatic reaction or transport into tissues becomes rate-limiting in the deficient state.

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