Increased Urinary Excretion of ^3H -Folic Acid Injected to Rats with Riboflavin Deficiency or Excessive Dietary Methionine*

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When rats were fed on either a riboflavin deficient diet or a high methionine diet, tritium labeled folic acid given intramuscularly was liable to be excreted into urine as such. It was assumed that this was partly due to a decreased activity of $\text{N}^{5,10}$ methylene-tetrahydrofolate reductase of the rat liver induced by either riboflavin deficiency or an excessive dietary methionine.

In a preceding paper of ours¹ it was reported that a marked decreased in $\text{N}^{5,10}$ methylene-tetrahydrofolate reductase activity associated with a considerable decrease in N^5 methyltetrahydrofolate transferase activity of the liver was observed in rats when fed on a riboflavin deficient diet for 26 days.

Furthermore, we² found that there was a tendency toward a decrease in a ratio of N^5 methyltetrahydrofolate to other folate compounds in the liver of the riboflavin deficient rats.

Recently Kutzbach and Stokstad³ have reported a feed back type regulation of $\text{N}^{5,10}$ methylene-tetrahydrofolate reductase by *s*-adenosylmethionine, tissue levels of which were closely related to methionine intake.

These data led to the present study which was designed to examine a difference, if any, in urinary excretion of radioactive materials after an intramuscular injection of tritium labeled folic acid followed by a flushing dose of non-labeled folic acid between rats fed on a riboflavin deficient, a high-methionine, or a control diet.

METHODS AND MATERIALS

Male rats of the Wistar strain, weighing 30 to 40 g, were used throughout this study. The rats were kept in individual cages with wire screen bottoms.

The composition of a control diet was shown in Table 1. A riboflavin deficient diet was that devoid of riboflavin from the control diet. A high-methionine diet⁴ was prepared by adding DL-methionine at a level of 2% to the control diet.

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TABLE 1. *Composition of a control diet for rats*

Ingredients	%
Casein (free form riboflavin)	20.0
Corn starch	40.0
Sucrose	27.0
Oil (soybean oil 80%, cod liver oil 20%)	8.0
Salt mixture*	4.0
Vitamin mixture†	0.85
Cholin chloride	0.15

* Salt mixture: CaCO_3 29.29%, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ 0.43%, KH_2PO_4 34.31%, NaCl 25.06%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 9.98%, $\text{Fe}(\text{C}_6\text{H}_5\text{O}_7) \cdot 6\text{H}_2\text{O}$ 0.623%, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.156%, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.121%, ZnCl_2 0.02%, KI 0.0005%, and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{27} \cdot 4\text{H}_2\text{O}$ 0.0025%

† Vitamin mixture: Thiamin hydrochloride 0.059%, riboflavin 0.059%, Ca pantothenate 0.235%, niacin 0.294%, pyridoxine hydrochloride 0.029%, menadione 0.006%, biotin 0.001%, folic acid 0.002%, inositol 1.176%, ascorbic acid 0.588%, hydroxocobalamin 0.0002%, and corn starch 97.551%.

The water was given *ad libitum*, but the amount of diets was limited to 10 g per rat per day.

Tritium labeled folic acid (potassium salt) was obtained from the Radiochemical Centre, Amersham, England. ^3H -folic acid dissolved in saline was used for injection.

Estimation of the radioactivity of urine specimens: 24-hour-urine specimens were collected in flasks containing 2 ml of 0.2 M phosphate buffer of pH 7.0 with ascorbic acid in a concentration of 1.0%. Twenty-five hundredths of the urine specimens was mixed with 10 ml of liquid scintillator.⁵ The mixture was centrifuged, a clear supernatant being measured for the radioactivity by a Packard tricarb automatic scintillation spectrometer operating at 5°C.

Chromatography of urinary folates: Urine specimens were adjusted to pH 7.0, to 20 ml of which were added 400 mg each of sodium ascorbate and charcoal. The mixture was kept at -4°C for one hour with occasional shaking, then centrifuged at 3,000 rpm for 5 minutes, and the supernatant was discarded. To the deposited charcoal 30 ml of a mixture consisting of ethanol, deionized water and concentrated NH_4OH (5:4:1, v/v) were added. The mixture was left stand for 20 minutes with constant stirring, then filtered. The filtrate added with 0.1 ml of 10% mercaptoethanol was evaporated at 40°C by a rotary evaporator. To the residue 5.0 ml of 1% ascorbate solution of pH 6.0 were added in order to extract folate compounds from the residue. The mixture was centrifuged, then a clear supernatant was subjected to chromatographic analysis for folates according to Silverman *et al.*'s method⁶ with a slight modification: Three g of DEAE cellulose, 1.4 g of hyflosuper cell, and 2.25 g of celite were suspended in 250 ml of 0.005N KOH solution. About 230 ml of the stirred slurry were packed by gravity flow into a glass tube (1.6×50 cm), then 35 ml of 0.5 N KOH were poured. Then the column was washed with deionized water until the rinse was neutral, then

with 35 ml of 0.5 M potassium phosphate buffer of pH 6.0, and then with deionized water until the rinse was free of phosphate. The final height of the column thus prepared was approximately 18 cm.

Four ml of the urinary folate extract were applied on the column, then followed with wash of 5 ml of 0.2% ascorbate solution of pH 6.0. A head of 40 ml of the 0.2% ascorbate solution of pH 6.0 was placed on the column and 150 ml of 0.5 M potassium phosphate buffer of pH 6.0 containing 0.2% ascorbate were passed, dropwise, through this head. Successive 5.0 ml fractions were collected. At last folates bound firmly to cellulose were washed out with 0.1 N NaOH solution.⁷

The radioactivity of each fraction obtained by the chromatography was estimated by a Packard tricarb automatic scintillation spectrometer.

The nature of folate compounds in the fractions was determined by comparison of their chromatographic behavior with that of synthetic folate compounds and by microbiological response to *Lactobacillus casei*, *Streptococcus faecalis* and *Pediococcus cerevisiae*.

N¹⁰ formyl-³H-folic acid was prepared according to Gordon *et al.*'s method,⁸ using ³H-folic acid and formic acid. ¹⁴C-methyl-N⁵-methyltetrahydrofolate was made by Rosenthal *et al.*'s method.⁹

A mixture containing pteridine derivatives and p-aminobenzoyl-glutamic acid was prepared by decomposition of tetrahydrofolic acid with trichloroacetic acid.

Pteridine derivatives¹⁰ were detected by the absorption at 365 mμ in 0.1 N NaOH, and p-aminobenzoyl-glutamic acid and tetrahydrofolic acid were detected with use of the Bratton-Marshall reaction.

Elution patterns of these folate compounds by the chromatography above mentioned were found as follows: Pteridine derivatives at No. 9; N¹⁰ formyltetrahydrofolic acid at No. 13; N¹⁰ formyldihydrofolic acid at No. 14; para-aminobenzoyl-glutamic acid at No. 15; N¹⁰ formyl folic acid at No. 17; N⁵ formyltetrahydrofolic acid at No. 22; N⁵ methyltetrahydrofolate at No. 23; tetrahydrofolate at No. 28; and dihydrofolic acid and pteroylglutamic acid at No. 42.

RESULTS AND DISCUSSION

On the 23rd day of the experimental feeding on the control, riboflavin deficient and high-methionine diets, ³H-folic acid, 200γ (80 μc) per kg of bodyweight, was injected intramuscularly to each of the rats, and 24-hour-urine specimens were collected and measured for the radioactivity. On the next day, 24 hours after the initial ³H-folic acid injection, non-labeled folic acid, 500γ per kg of bodyweight, was injected intramuscularly to each of the rats, 24-hour-urine specimens being collected again for estimation of the radioactivity and chromatographic analysis of radioactive materials.

The rate of urinary excretion of radioactivity in the 24-hour-urine after a flushing dose was found to be higher in rats with riboflavin deficiency and those fed on a high-methionine diet than in control rats (cf. Table 2 and Fig. 1).

TABLE 2. Urinary excretion of radioactivity after an intramuscular injection of ^3H -folic acid and that after a flushing dose of non-labeled folic acid given on the next day in rats with various conditions

Group	No. of rats	Body weight (g)	Dose of ³ H-folic acid given intramuscularly (cpm)	Excretion of radioactivity in 24-hour-urine				Total of radioactivity excreted in urine for 2 days (% of ³ H-folic acid injected)
				First day* (³ H-folic acid was injected)		Second day* (Non-labeled folic acid was injected)		
				cpm	% of a dose of ³ H-folic acid injected	cpm	% of a dose of ³ H-folic acid injected	
Control diet	6	125	358.0 × 10 ⁴	305,384	8.5	21,136	0.6	9.1
	7	128	366.6 × 10 ⁴	213,512	5.8	37,376	1.0	6.8
	8	125.5	359.4 × 10 ⁴	323,720	8.7	21,992	0.6	9.3
B ₂ deficient diet	16	51	145.9 × 10 ⁴	124,968	8.5	35,760	2.5	11.0
	17	53	151.8 × 10 ⁴	155,728	10.2	41,632	2.7	12.7
	18	50	143.2 × 10 ⁴	119,072	8.3	13,640	1.0	9.3
High-methio-nine diet	23	109	312.2 × 10 ⁴	637,536	20.4	108,144	3.5	23.9
	24	105.5	302.2 × 10 ⁴	372,928	12.3	55,144	1.8	14.1
	25	113	313.6 × 10 ⁴	496,880	15.8	88,536	2.8	18.6

* On the first day of the experiment, ^3H -folic acid, 200 γ (80 μc)/kg, was injected intramuscularly, and on the second day non-labeled folic acid, 500 γ /kg, was given intramuscularly.

Fig. 1. Urinary excretion of radioactivity after an intramuscular injection of ^3H -folic acid, 200 γ (80 μc) per kg. Next day a flushing dose of non-labeled folic acid, 500 γ per kg, was injected intramuscularly.

○—○ Rats fed on a control diet
 △---△ Rats fed on a B_2 -deficient diet for 23 days
 x---x Rats fed on a control diet to which D-L methionine was added in a concentration of 2%

Fig. 2. Distribution of the radioactivity in the chromatograms of urine specimens of rats. The urine specimens were collected after a flushing dose of non-labeled folic acid 24 hours after ³H-folic acid injection.

The distribution of the radioactivity in the chromatogram of urine specimens was shown in Fig. 2, indicating that there were three peaks of the radioactivity ('a', 'b' and 'c' in Fig. 3) in any of urine specimens obtained from rats of the control, riboflavin deficient and high-methionine groups.

Judging from the elution patterns of peaks 'a', 'b' and 'c' in the chromatograms it was assumed that the peak 'a' consisted of para-aminobenzoyl-glutamic acid, N¹⁰ formyl-dihydrofolic acid and N¹⁰ formyltetrahydrofolic acid; the peak 'b' consisted of N⁵ formyltetrahydrofolic acid and N⁵ methyltetrahydrofolic acid; and the peak 'c' consisted of dihydrofolic acid and pteroylglutamic acid.

The percentage distribution of the radioactivity at peaks 'a', 'b' and 'c' in

TABLE 3. Distribution of radioactive materials in 24-hour-urine of rats collected after a flushing dose of non-labeled folic acid injection. The initial ³H-folic acid injection was done 24 hours prior to the flushing dose

Diet	Control diet			B ₂ deficient diet			High-methionine diet		
Pattern	Nos. of tube	cpm	%	Nos. of tube	cpm	%	Nos. of tube	cpm	%
Peak 'a'	13 15	7,330	46.1	13 18	8,436	36.3	11 18	22,144	37
Peak 'b'	21 24	4,980	31.4	22 25	2,200	9.5	20 23	6,766	11
Peak 'c'	41 45	3,582	22.5	42 44	12,616	54.2	42 44	30,786	52
Total		15,892	100		23,252	100		59,696	100

urine from riboflavin deficient, high-methionine and control groups was given in Table 3.

From Table 3 it was apparent that a decrease in the radioactivity of the peak 'b' associated with an increase in the radioactivity of the peak 'c' was observed in rats with riboflavin deficiency and those fed on a high-methionine diet.

The present findings of an increase in the radioactivity at the peak 'c' associated with a decrease in that of the peak 'b' were found to be common to both the cases of riboflavin deficient rats and of rats fed on a high-methionine diet, and were explained on the basis of a decreased activity of N^{5,10} methylenetetrahydrofolate reductase of the rat liver induced by either riboflavin deficiency or a high-methionine feeding.

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