Determination of Folate Derivatives in Rat Tissues during Folate Deficiency

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Summary A method for the sensitive and specific determination of folate derivatives was developed. The method involves hydrolysis by γ-glutamyl hydrolase and high-performance liquid chromatography with electrochemical detection. The method was applied to measure the change in the level of folate derivatives in the liver, kidney, spleen and brain of rats during folate deficiency. 5,6,7,8-Tetrahydrofolic acid was the major folate derivative in the liver, kidney, spleen and brain. Total concentration of folate derivatives decreased from the second week of folate deficiency in the liver, kidney, spleen and brain followed by anemia, which appeared at the fifth week. The level of 5,6,7,8-tetrahydrofolic acid in the brain did not change during folate deficiency, but it significantly decreased in the liver, kidney and spleen.

Key Words folate, folate deficiency

Folate is a water soluble vitamin that plays an important role in the biosynthesis of thymidylate, purine and amino acid, and is essential for DNA synthesis and cell proliferation (1). Folate deficiency results in decreased rates of new blood cell production that in turn leads to the typical clinical manifestation of megaloblastic anemia (2). Although many studies of folate deficiency have been performed in relation to anemia, few studies are available regarding the quantity and metabolism of folates in mammals. One reason may be ascribed to the fact that no convenient method to determine folates is available. The quantitation of each folate is difficult, because folates in tissue exist at very low levels and are unstable. Early studies of folate determination employed bioassay using microorganisms such as Lactobacillus casei, Streptococcus faecalis and Pediococcus cerevisiae (3–7). However, this method is time consuming, and cannot specifically determine each folate derivative. Recently, methods using high-performance liquid chromatography (HPLC) have been introduced for the separation of folate derivatives based on anion-exchange, ion-pair and reversed-phase chromatography (8–17). However, determination of all folate compounds in the liver remains incomplete.

In the present study, we developed a specific and sensitive method for the determination of folates and systematically estimated changes in the levels of major folate derivatives in animal tissues during folate deficiency for the first time.

MATERIALS AND METHODS

Materials. Pteroylglutamate (PteGlu) was purchased from Sigma (St. Louis, MO, USA). 5-Formyltetrahydrofolic acid (5-CHO-THF) was obtained from Fluka BioChemika (Buchs, Switzerland). 5,6,7,8-Tetrahydrofolic acid (THF), 5-methyl-(6R,S)-5,6,7,8-tetrahydrofolic acid calcium salt (5-CH3-THF) and 7,8-dihydrofolic acid (DHF) were purchased from Dr. B. Schircks Laboratory (Jona, Switzerland). The concentration of standard folate derivatives was determined by absorption spectrum (18, 19). All other reagents were purchased from Wako Pure Chem. Co., Ltd. (Osaka, Japan) and were of analytical grade.

Animals and diets. Care and treatment of experimental animals during this experiment were followed by guidelines from the Prime Minister’s Office of Japan (No. 6 of March 27, 1980). Male Wistar rats at 3-wk-old were purchased from Japan SLC Ltd. (Hamamatsu, Shizuoka, Japan). Two groups of rats were housed in a room at a temperature 24±2°C, with a 12 h light-dark cycle and were given the test diet and water ad libitum at all times. The folate-deficient rats received an amino acid-defined, and folate-deficient diet (20), 1 kg of which contained 178 g amino acid mixture, 404 g dextrin, 203 g sucrose, 100 g corn oil containing 0.015% 2,6-butylation hydroxytoluene (BHT), 10 g vitamin mixture without folic acid (21), 50 g mineral mixture (21) and 2 g choline chloride as shown in Table 1. The control group was fed the same diet supplemented with 8 mg folate/kg diet. All of the diets contained 1% (W/W) succinylsulfathiazole to repress intestinal microfloral folate production. Animals were weighed every day. The rats were killed under diethyl ether anesthesia at the 2, 4, 5 and 6 wk, and the liver, kidney, spleen and brain were excised. The excised tissues were frozen immediately in liquid nitrogen, and then stored at −80°C until use. Blood hematocrit was measured by centrifugation (3,000 rpm, 30 min) and blood hemoglobin concentration was measured using a diagnostic kit (Hemoglobin Test Wako, Wako Pure Chem. Co., Ltd., Osaka, Japan).

Extraction of folate from the tissue. Frozen tissue was sliced in liquid nitrogen, to which was added 2 vol of boiling extraction solution (0.2% (W/V) of sodium ascorbate containing 1 mM dithiothreitol (pH 7.8)) in a glove box filled with nitrogen gas. After sealing with a serum cup, the sample was placed in a boiling water bath for 10 min and then rapidly cooled on ice. After
Table 1. Diet composition.

| Component (g/kg diet) |  |
|----------------------|---|
| Amino acid mixture<sup>1)</sup> | 178 |
| Cellulose            | 50  |
| Corn oil             | 100 |
| Dextrin              | 404 |
| Sucrose              | 203 |
| Vitamin mixture<sup>2)</sup> | 10 |
| Mineral mixture      | 50  |
| Choline chloride     | 2   |
| Succinylsulfathiazole| 10  |

1) Amino acid mixture provided the following amounts per kilogram diet: DL-alanine 7.12 g, L-arginine 11.4 g, L-asparagine 6.10 g, L-cystine 3.56 g, L-glutamic acid 35.6 g, L-glycine 23.7 g, L-histidine 3.36 g, L-isoleucine 8.34 g, L-leucine 11.3 g, L-lysine 14.6 g, L-methionine 8.34 g, L-phenylalanine 11.8 g, L-proline 3.56 g, L-serine 3.56 g, L-threonine 8.34 g, L-tryptophan 1.77 g, L-tyrosine 3.56 g, L-valine 8.34 g.

2) The folate-deficient diet used a folate-free vitamin mixture. The control diet used a vitamin mixture containing 8 mg folate/kg diet.

adding 2 vol of the extraction solution, the sample was homogenized with a teflon homogenizer under nitrogen atmosphere. To the sample was added an equal volume of dichloromethane to remove lipid. After shaking and centrifugation (3,000 rpm, 4°C, 10 min), the supernatant was lyophilized overnight. The lyophilized sample was dissolved in 300 μL of the extraction solution, to which was added 30 μL of γ-glutamyl hydrolase prepared from rat kidney. The sample was reacted for 1 h at 37°C under a nitrogen atmosphere to hydrolyze the folylpolyglutamates to folylmonoglutamates. The sample was placed in a boiling water bath for 5 min and then cooled in an ice bath. The precipitates were removed by centrifugation at 10,000 × g for 5 min, and 10 μL of the supernatant was directly applied to the HPLC analysis.

**HPLC analysis of folate derivatives.** A Shimadzu LC-10A pump (Kyoto, Japan) was used. The sample was applied to a μBondasphere-5-phenyl 100A column (3.9×150 mm, Waters Ltd., Tokyo, Japan). The mobile phase was a solution of 50 mM potassium dihydrogen phosphate (pH 3.5) containing 0.1 mM ethylenediamine tetraacetic acid disodium salt, dihydrate (EDTA) and 8% methanol, and the flow rate was 1 mL/min. Detection was made using an amperometric detector ICA-3062 (Toa Electronics Ltd., Japan) at the potential of +750 mV versus Ag/AgCl reference electrode.

Preparation of γ-glutamyl hydrolase from rat kidney. γ-Glutamyl hydrolase [EC. 3.4.22.12] used to hydrolyze folylpolyglutamate was prepared from rat kidney. Rat kidneys were homogenized with 5 vol of potassium phosphate buffer (pH 6.0) containing 10 mM 2-mercaptoethanol, 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 36,000 × g for 30 min at 4°C, the supernatant was collected. The crude solution was brought to 30–70% (NH₄)₂SO₄ fractionation. The precipitate was dissolved in a small amount of 20 mM potassium phosphate buffer, pH 6.0, containing 10 mM 2-mercaptoethanol, and dialyzed against the buffer overnight at 4°C. The dialyze was centrifuged at 36,000 × g for 15 min, and the clear supernatant was adjusted to pH 4.5 with 0.5 M acetic acid. After centrifugation, the supernatant was adjusted to pH 7.3 with 1 N NaOH, and then applied to a column of methotrexate affinity which had been previously equilibrated with 50 mM Tris-HCl buffer, pH 7.3, containing 10 mM 2-mercaptoethanol. The affinity gel was prepared by coupling the carboxy groups of methotrexate to the free amino groups of AH-sepharose 4B by the carbodiimide (22). The column was eluted with the above buffer. The fractions containing enzyme activity were collected and (NH₄)₂SO₄ was adjusted to 80% saturation. After 30 min, the precipitate was collected by centrifugation for 30 min and dissolved in 50 mM Tris-HCl buffer, pH 7.3, containing 10 mM 2-mercaptoethanol. The γ-glutamyl hydrolase activity was determined by measuring the formation of PteGlu from pteroyltriglutamate (PteGlu₃) utilizing HPLC (column: μBondasphere 5C₁₈, mobile phase: 50 mM tetra-n-butylammonium phosphate containing 40% methanol). This enzyme preparation did not show any DHF reductase activity by spectrophotometrical assay (23). Partially purified γ-glutamyl hydrolase was stored at −80°C until use.

**Statistical analysis.** The data were expressed as mean±SD and analyzed for significance by Student’s t-test.

**RESULTS**

**Change of body weight of rats during folate deficiency**

Changes in the body weights of rats are shown in Fig. 1. The body weight of rats on the starting day (day 0) was 62.6±5.4 g. Body weights of the control group fed with folate-containing diet and the folate-deficient rat group both increased steadily. After 3 wk of folate defi-
Change in Folates during Folate Deficiency in Rats

Fig. 2. Effect of folate deficiency on blood hematocrit. Data are expressed as means±SD (n=5-8). **: Significantly different from the corresponding control (p<0.01).

Fig. 3. Effect of folate deficiency on hemoglobin concentration. Data are expressed as means±SD (n=5-8). **: Significantly different from the corresponding control (p<0.01).

The body weight gain of the folate-deficient rats was lower than that of the control rats, and the body weight of the folate-deficient group (176.7±15.1 g) was significantly different from that of the control group (192.4±10.4 g). After 5 wk, the body weight of the folate-depleted animals began to decrease. After 6 wk, the body weight of the folate-deficient animals was 171.9±20.0 g, which was significantly lower than that (303.2±15.7 g) of the control group.

Change in blood hematocrit and hemoglobin concentration

The hematocrit values of the folate-deficient group were the same as the control group until 4 wk as shown in Fig. 2. After 5 wk of folate deficiency, hematocrit value decreased to 27.8±8.0%, which was significantly lower than that of the control group (44.5±1.2%). After 6 wk, the value for folate-depleted rats fell to 13.2±6.8%.

After 2 and 4 wk of folate deficiency, the hemoglobin concentration of the folate-deficient group was 12.6±0.5, 14.2±0.92 g/100 mL, respectively, and these values were similar to the corresponding control value as shown in Fig. 3. After 5 and 6 wk of folate deficiency, the concentration of hemoglobin had declined significantly to 9.45±2.69 and 4.54±2.54 g/mL, respectively. These results indicate that anemia developed 5 and 6 wk after the induction of folate deficiency.

Separation of standard folate derivatives by HPLC

The HPLC profile of standard folate derivatives is shown in Fig. 4. THF, 5-CH$_3$-THF, 5-CHO-THF, PteGlu and DHF were detected. The detection limit was 1 pmol per injection for THF, 5-CH$_3$-THF or 5-CHO-THF, and that for PteGlu or DHF was 10 pmol per injection. For the determination of folate derivatives in rat tissues, a recovery experiment was made first using the standard folate derivatives. When 10 nmol of each standard folate derivative was added to liver homogenate, recovery for THF, 5-CH$_3$-THF, 5-CHO-THF and PteGlu was 80 to 90%. DHF was recovered by about 60% (Table 2).

Determination of folate derivatives in rat tissues during folate deficiency

Liver. The HPLC profile of folate derivatives extracted from liver is shown in Fig. 5. The liver of the
control animals fed with folate contained THF, 5-CH$_3$-THF, 5-CHO-THF, PteGlu and DHF as shown in Table 3. The relative contents of THF, 5-CH$_3$-THF and 5-CHO-THF in the liver of the 2-wk-old control rat were about 53, 43 and 3.4% of total folate, respectively. After 2 wk of folate deficiency, the level of THF was 3.93±0.86 nmol/g, which was significantly lower than that of the controls (8.14±0.98 nmol/g) (Table 3). After 4, 5 and 6 wk of folate deficiency, the level of THF was similar to that of folate-deficient animals at the 2 wk. The level of 5-CH$_3$-THF after 2 wk decreased significantly to 45% of the corresponding controls. After 4, 5 and 6 wk of folate deficiency, the content of 5-CH$_3$-THF in the liver fell significantly to 30% of the corresponding control level. The level of 5-CHO-THF in the liver at 2 and 4 wk of deficiency declined to 46 and 50% of the corresponding controls, respectively. After 5 and 6 wk of folate deficiency, 5-CHO-THF in the liver decreased to 3% of the control level. PteGlu was a very small component of the total folate in the liver, The concentrations of PteGlu of folate-deficient rat liver tended to be lower than those of the control rats, but these differences were not significant (Table 3).

**Kidney.** In the kidney, three folate derivatives were detected as shown in Table 4. The most abundant folate derivative was THF, which amounted to about 70% of total folate in the kidney of the 2-wk-old control rat. The relative contents of 5-CH$_3$-THF and 5-CHO-THF were about 23 and 6.8% of total folate, respectively. After 2 and 4 wk of folate deficiency, THF in the kidney decreased to 32 and 46% of that in the corresponding control kidney, respectively. After 5 wk, THF in the kidney declined even more, to 17% of the controls. After 6 wk, the level of THF decreased to below the detection limit. The 5-CH$_3$-THF concentration in the kidney of the folate-deficient rat was not significantly different from that of the corresponding control rat. The levels of 5-CHO-THF in the kidney after 2, 4, 5 and 6 wk of folate deficiency were 23, 16, 6 and 8.2% of the corresponding control level, respectively, and these values were significantly lower than the control values (Table 4).

**Spleen.** The major folate derivative in the spleen was also THF, which amounted to 76% of total folate of the 2-wk-old control rat as shown in Table 5. The relative contents of 5-CH$_3$-THF, 5-CHO-THF, PteGlu and DHF were about 17, 4.1, 1.6 and 1.1% of total folate, respectively. After 2 wk of folate deficiency, the level of THF decreased significantly to 23% of the control level. After 4 and 5 wk, THF decreased even more significantly to 9 and 5.8% of the corresponding control value, respectively. After 6 wk of deficiency, the THF level dropped below the detection limit. The concentration of 5-CH$_3$-THF and 5-CHO-THF declined during folate deficiency. However, these values were not significantly different from the corresponding control value. The levels of both PteGlu and DHF after the depletion decreased below the detection limit (Table 5).

**Brain.** THF, 5-CH$_3$-THF, 5-CHO-THF and PteGlu were detected in the brain as shown in Table 6. The relative contents of THF, 5-CH$_3$-THF, 5-CHO-THF and PteGlu were about 47, 48, 1.2 and 3.3% of total folate in the brain of the 2-wk-old control rat, respectively. After 2, 4, 5 and 6 wk of folate deficiency, the level of

| Treatment | THF | 5-CH$_3$-THF | 5-CHO-THF | PteGlu | DHF |
|-----------|-----|--------------|-----------|--------|-----|
| Control   |     |              |           |        |     |
| 2 wk      | 8.14±0.98 | 6.66±1.33   | 0.52±0.13 | 0.09±0.21 | ND |
| 4 wk      | 7.04±1.54 | 6.30±1.94   | 0.46±0.27 | 0.49±0.79 | ND |
| 5 wk      | 6.53±1.31 | 6.06±0.69   | 0.91±0.41 | 0.58±0.52 | ND |
| 6 wk      | 6.94±1.76 | 7.21±1.55   | 1.01±0.85 | 0.33±0.20 | 0.04±0.05 |
| Deficiency|     |              |           |        |     |
| 2 wk      | 3.93±0.86** | 2.98±0.82** | 0.24±0.16* | 0.02±0.03 | ND |
| 4 wk      | 3.16±1.16** | 1.89±0.55** | 0.23±0.12 | 0.02±0.04 | ND |
| 5 wk      | 3.66±0.93** | 1.76±0.87** | 0.03±0.05* | 0.01±0.02 | 0.004±0.01 |
| 6 wk      | 4.01±0.80*  | 1.86±0.41** | 0.03±0.09 | 0.02±0.06 | ND |

Values are means±SD (n=5–8). ND: Not detected.

*: **: Significantly different from the corresponding control (*: p<0.05, **: p<0.01).
Table 4. Effect of folate deficiency on folate content in rat kidney.

| Treatment | Folate content (nmol/g kidney) | THF | 5-CH$_3$-THF | 5-CHO-THF | PteGlu | DHF |
|-----------|--------------------------------|-----|--------------|-----------|--------|-----|
|           |                                |     |              |           |        |     |
| Control   |                                |     |              |           |        |     |
| 2 wk      | 6.22±0.21                      | 2.08±0.97 | 0.61±0.45 | ND       | ND    |     |
| 4 wk      | 4.64±1.57                      | 3.22±0.89 | 0.63±0.12 | ND       | ND    |     |
| 5 wk      | 4.74±2.33                      | 1.65±0.26 | 0.50±0.15 | ND       | ND    |     |
| 6 wk      | 3.37±0.90                      | 2.09±0.09 | 0.49±0.14 | ND       | ND    |     |
| Deficiency|                                |     |              |           |        |     |
| 2 wk      | 1.99±0.62*                     | 0.99±1.07 | 0.14±0.09** | ND       | ND    |     |
| 4 wk      | 2.12±1.89*                     | 1.04±2.10 | 0.10±0.07** | ND       | ND    |     |
| 5 wk      | 0.79±0.25*                     | 1.10±1.88 | 0.03±0.03** | ND       | ND    |     |
| 6 wk      | ND                             | 1.41±1.26 | 0.04±0.05** | ND       | ND    |     |

Values are means±SD (n=3–5).
ND: Not detected.
*, **: Significantly different from the corresponding control (*: p<0.05, **: p<0.01).

Table 5. Effect of folate deficiency on folate content in rat spleen.

| Treatment | Folate content (nmol/g spleen) | THF | 5-CH$_3$-THF | 5-CHO-THF | PteGlu | DHF |
|-----------|--------------------------------|-----|--------------|-----------|--------|-----|
|           |                                |     |              |           |        |     |
| Control   |                                |     |              |           |        |     |
| 2 wk      | 3.38±0.95                      | 0.76±0.83 | 0.18±0.25 | 0.07±0.01 | 0.05±0.06 |
| 4 wk      | 2.10±1.35                      | 0.85±0.50 | 0.05±0.05 | 0.10±0.13 | 0.03±0.01 |
| 5 wk      | 2.25±1.16                      | 0.83±0.51 | 0.08±0.08 | 0.38±0.31 | 0.03±0.02 |
| 6 wk      | 2.37±0.17                      | 1.35±0.71 | 0.14±0.10 | 0.39±0.13 | 0.05±0.07 |
| Deficiency|                                |     |              |           |        |     |
| 2 wk      | 0.79±0.61**                    | 0.43±0.44 | 0.03±0.02 | ND       | ND    |     |
| 4 wk      | 0.19±0.26**                    | 0.63±0.65 | 0.02±0.03 | ND       | ND    |     |
| 5 wk      | 0.13±0.15**                    | 0.37±0.47 | 0.03±0.03 | ND       | ND    |     |
| 6 wk      | ND                             | 0.33±0.36 | 0.09±0.08 | ND       | ND    |     |

Values are means±SD (n=3–6).
ND: Not detected.
**: Significantly different from the corresponding control (p<0.01).

Table 6. Effect of folate deficiency on folate contents in rat brain.

| Treatment | Folate content (nmol/g brain) | THF | 5-CH$_3$-THF | 5-CHO-THF | PteGlu | DHF |
|-----------|--------------------------------|-----|--------------|-----------|--------|-----|
|           |                                |     |              |           |        |     |
| Control   |                                |     |              |           |        |     |
| 2 wk      | 1.14±0.13                      | 1.16±0.19 | 0.03±0.02 | 0.08±0.08 | ND    |
| 4 wk      | 1.26±0.47                      | 1.78±1.51 | 0.05±0.04 | 0.16±0.14 | ND    |
| 5 wk      | 1.72±1.43                      | 1.87±0.82 | 0.06±0.06 | 0.10±0.03 | ND    |
| 6 wk      | 2.22±1.47                      | 1.50±1.12 | 0.03±0.03 | 0.25±0.23 | ND    |
| Deficiency|                                |     |              |           |        |     |
| 2 wk      | 1.13±0.30                      | 0.26±0.08* | 0.03±0.01 | 0.11±0.03 | ND    |
| 4 wk      | 1.11±0.19                      | 0.33±0.03 | 0.09±0.04 | 0.26±0.11 | ND    |
| 5 wk      | 1.26±0.66                      | 0.32±0.12* | 0.05±0.04 | 0.17±0.15 | ND    |
| 6 wk      | 1.28±0.49                      | 0.38±0.33* | 0.03±0.04 | 0.15±0.08 | ND    |

Values are means±SD (n=3–4).
ND: Not detected.
*: Significantly different from the corresponding control (p<0.05).
THF showed no decrease from the corresponding control level. After 2, 4, 5 and 6 wk, the level of 5-CH₃-THF declined significantly to 20% of that of the corresponding controls. The levels of 5-CHO-THF and PteGlu did not change significantly during folate deficiency. The content of DHF in the brain was below the detection limit (Table 6).

**DISCUSSION**

Folate derivatives are very susceptible to air oxidation (24). Therefore, it is important to protect folate derivatives from oxidation during extraction. Ascorbate solution afforded effective protection at lower concentrations, and ascorbate solution was the agent of choice for protecting the labile reduced folates during extraction and assay procedures (25). However, Wilson and Horne (25) demonstrated that extraction of folate derivatives with ascorbate solutions at high temperature led to chemical interconversion. They also showed that sodium ascorbate solution used at elevated temperatures was not suitable for extracting tissues for subsequent assay of individual folate derivatives. Although we used heated ascorbate solution for extraction, each folate derivative could be recovered without chemical interconversion. In our method, all the procedures were carried out under a nitrogen atmosphere, and folate derivatives were extracted using 2% (W/V) sodium ascorbate solutions buffered at pH 7.8 with the addition of 1 mM dithiothreitol. It is conceivable that a nitrogen atmosphere and the presence of dithiothreitol allow the recovery of folate derivatives at a good yield without chemical interconversion.

In the liver, the major folate derivative was THF and 5-CH₃-THF, being consistent with previous studies (8, 11, 26, 27). McMartin et al. (11) reported that no endogenous PteGlu and DHF were present in the liver. Wilson and Horne (15) and Duch et al. (8) did not report the presence of PteGlu and DHF in the liver. In the present study, PteGlu and DHF were detected in very small quantities in the liver. We could determine PteGlu and DHF also in the spleen, and PteGlu in the brain. Our method involves the removal of lipid with dichloromethane under a nitrogen atmosphere and lyophilization. These concentration steps under reduced conditions seems to make it possible to detect low levels of folate derivatives such as PteGlu and DHF. Furthermore, we used partially purified γ-glutamyl hydrolase from the rat kidney. This enzyme preparation did not show any DHF reductase activity. The folate derivatives in mammalian tissue exist primarily as polyglutamates (26, 28), and analysis of these polyglutamates is usually performed after hydrolysis to the corresponding monoglutamate using γ-glutamyl hydrolase. When we used a crude enzyme preparation from the rat kidney for hydrolyzing pteroylpolyglutamate, we could not detect DHF in the rat liver, but could detect more THF than the real quantity in the liver. McMartin et al. (11) used partially purified hog kidney γ-glutamyl hydrolase, and Wilson and Horne (15) used γ-glutamyl hydrolase prepared from rat plasma by simply dialyzing against a buffer containing charcoal. It is probable that their enzyme preparations contained DHF reductase, and they could not detect DHF.

In this paper, we report, for the first time, the changes in the levels of folate derivatives in animal tissue during folate deficiency using our specific method. The folate derivatives in the liver, kidney, spleen and brain decreased from 2 wk after introducing deficiency, and anemia appeared at 5 wk. The distribution and changes in the level of folate derivatives differed in each animal tissue during folate deficiency. In the brain, the level of THF, which was the major folate derivative in the brain, did not change during folate deficiency, while THF in the liver, kidney and spleen showed a significant decrease. The level of folate in the brain was retained during deficiency and decreased at a slow rate. The method developed in this study is rapid and simple, and is very specific and sensitive for each folate derivative with no interconversion. This method may be useful to study folate metabolism and its regulation in mammalian tissue.

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**REFERENCES**

1) Baugh CM, Krumdieck CL. 1997. Naturally occuring folates. Ann NY Acad Sci 86: 7–28.
2) Antony AC. 1991. Basic principles and practice. In: Hematology (Hogmann R, Benz EJ, Shattil SJ, Furie B, Cohen HJ, eds), Churchill Livingstone, New York.
3) Bird OD, McGlohon VM, Vaitkus JW. 1965. Naturally occurring folates in the blood and liver of the rat. Anal Chem 12: 18–35.
4) Bird OD, McGlohon VM, Vaitkus JW. 1969. A microbiological assay system for naturally occurring folates. Can J Microbio 15: 465–472.
5) Bird OD, McGlohon VM. 1972. Differential assays of folic acid in animal tissues. In: Analytical Microbiology (Kavanaugh F, eds), Accademic Press, New York.
6) Donaldson DD, Keresztesy JC. 1957. Naturally occurring forms of folic acid. J Biol Chem 234: 3235–3240.
7) Silverman M, Law JW, Kaufman B. 1961. The determination of folic acid activities in liver of leukemic cells of the mouse. J Biol Chem 236: 2530–2533.
8) Duch DS, Bowers SW, Nichol CA. 1983. Analysis of folic cofactor levels in tissues using high-performance liquid chromatography. Anal Biochem 130: 385–392.
9) Horne DW, Briggs WT, Wanger C. 1981. High-pressure liquid chromatographic separation of the naturally occurring folic acid monoglutamate derivatives. Anal Biochem 116: 393–397.
10) Lankema J, VanderKleins E, Jansen MJ, Th. 1980. Determination of 5-methyltetrahydrofolic acid in plasma and spinal fluid by high performance liquid chromatography, using on-column concentration and electrochemical detector. J Chromatogr 182: 35–45.
11) McMartin KE, Virayotha V, Tephly TR. 1981. High-pressure liquid chromatography separation and determination of rat liver folates. Arch Biochem Biophys 209:
12) McNulty H, McPartlin J, Weir D, Scott J. 1993. Reversed-phase high-performance liquid chromatographic method for the quantitation of endogenous folate catabolites in rat urine. J Chromatogr 614: 59–66.
13) Selheb J. 1989. Determination of tissue folate composition by affinity chromatography followed by high-pressure ion pair liquid chromatography. Anal Chem 182: 84–93.
14) Silan L, Jadaud P, Whitfield LR, Wainer IW. 1990. Determination of low levels of the stereoisomers of leucovorin and 5-methyltetrahydrofolate in plasma using a coupled chiralachiral high-performance liquid chromatographic system with post-chiral column peak compression. J Chromatogr 532: 227–236.
15) Wilson SD, Horne DW. 1984. High-performance liquid chromatographic determination of the distribution of naturally occurring folic acid derivatives in the liver. Anal Biochem 142: 529–535.
16) Tani M, Iwai K. 1983. High chromatographic separation of physiological folate monoglutamate compounds. Investigation of absorption and conversion pteroylglutamic acid in the small intestine of the rat in situ. J Chromatogr 267: 175–181.
17) Kohashi M, Inoue K, Sotobayashi H, Iwai K. 1986. Microdetermination of folate monoglutamates in serum by liquid chromatography with electrochemical detection. J Chromatogr 382: 303–307.
18) Gupta VS, Huennekens FM. 1967. Preparation and properties of crystalline 5-methyl tetrahydrofolate and related compounds. Arch Biochem Biophys 120: 712–718.
19) Uyeda K, Rabinowitz JC. 1965. Metabolism of formiminoglycine. J Biol Chem 240: 1701–1710.
20) Walzem RL, Clifford AJ. 1988. Folate deficiency in rats fed diets containing free amino acids or intact proteins.
21) American Institute of Nutrition. 1997. Report of American Institute of Nutrition ad hoc committee on standards for nutritional studies. J Nutr 107: 1340–1348.
22) Kaufman BT, Pierce JV. 1971. Purification of dihydrofolic reductase from liver by affinity chromatography. Biochem Biophys Res Commun 44: 608–613.
23) Gunderson LE, Dunlop RB, Harding NGL, Freisheim JH, Otting F, Huennekens FM. 1972. Dihydrofolate reductase from amethopterin-resistant Lactobacillus casei. Biochemistry 11: 1018–1022.
24) O’Broin, Temperly JD, Brown JP, Scott JM. 1975. Nutritional stability of various naturally occurring monoglutamate derivatives of folic acid. Am J Clin Nutr 28: 438–444.
25) Wilson SD, Horne DW. 1983. Evaluation of ascorbic acid in protecting labile folic acid derivatives. Proc Natl Acad Sci USA 80: 6500–6504.
26) Eto I, Krumdiek CL. 1982. Determination of three different pools of reduced one-carbon-substituted folates. III. Reversed-phase high-performance liquid chromatography of the azo dye derivatives of p-aminobenzoxypolyglutamate and its application to the study of unlabeled endogenous pteroylpolyglutamates of rat liver. Anal Biochem 120: 323–329.
27) Gregory JE, Sartain PB, Day BPF. 1984. Fluorometric determination of folacin in biological materials using high performance liquid chromatography. J Nutr 114: 341–353.
28) Eto I, Krumdiek CL. 1981. Determination of three different pools of reduced one-carbon-substituted folates. II. Quantitation and chain length determination of pteroylpolyglutamates of rat liver. Anal Biochem 115: 138–146.